Attorney Docket No. 31242-701.201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application	
Inventor(s): Elizabeth Moyer et al.	Confirmation No.: 2967
Application No.: 09/393,590	Group Art Unit: 1645
Filed: September 9, 1999	Examiner: DEVI, S.J.N.
Title: STABLE LIQUID FORMULATIONS OF BOTULINUM TOXIN	

DECLARATION UNDER 37 CFR 1.132

M/S Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Elizabeth Moyer, a joint-inventor of the above-referenced application, hereby declare that:

1. I am a joint inventor of the invention claimed in the above-referenced application. My curriculum vitae is provided as Exhibit 1. I have read the above-referenced application including the claims being presented in the Supplemental Response accompanying this declaration, as well Melling, J. et al., Eye 2:16-23 (1988) [Exhibit 2]; Sacks, H. and Covert, S.V., App. Microbiol. 28:374-382 (1974) [Exhibit 3]; Ohishi, I. and Sakaguchi, G., App. Microbiol. 28:923-928 (1974) [Exhibit 4]; Ohishi, I. and Sakaguchi, G., Infect Imm. 28:303-309 (1980) [Exhibit 5]; Ohishi, I. et al., Infect. Imm. 30:668-673 (1980) [Exhibit 6]; Schiavo, G. et al., J. Biol. Chem. 269:20213-20216 (1994) [Exhibit 7]; Lachman, L. et al. *In*: The Theory and Practice of Industrial Pharmacy 3rd edition (1986) [Exhibit 8]; and I am familiar with their contents.

2. I am familiar with the knowledge of one of skill in the art at the time of filing of the above-referenced application. At the time of filing of the application, one of skill in the art would have been familiar with the purification of botulinum toxin serotypes, and its use in treating a variety of neuromuscular disorders. In addition, one of ordinary skill would also realize that the purity of the botulinum toxin serotypes is important in the pharmaceutical treatment of patients, as well as in the stability of a botulinum toxin pharmaceutical formulation. As outlined in the article by Melling et al. [Exhibit 2], the preparation of botulinum toxin requires the following of rigorous standards "for a product to be acceptable and licensable for clinical use." *See* Melling et al., pg. 20, col. 1, 3rd full paragraph. Melling et al. outlines the purification steps necessary for preparation of botulinum toxin for clinical use:

> *Purification*. The precipitated crude toxin is re-dissolved and purified by a series of procedures involving ammonium sulphate precipitation and *ion-exchange chromatography* (Table IV). During these procedures the process is monitored for the absence of contamination with extraneous micro-organisms as well as the toxicity and protein content of the extracts.

Melling et al., pg. 20, col. 2, 1st full paragraph (emphasis added). Table IV lists two ion-exchange chromatography media, including DEAE Sephadex A50 for batch preabsorption and DEAE-Sephacel ion-exchange chromatography. *See Id.* at Table IV, pg. 21. In contrast to the references cited by the Examiner, the purification of clinical-grade botulinum toxin only used ammonium sulfate precipitation as a purification means. Such impure preparations would contain contaminants, including proteases and nucleic acids that could hasten degradation of the botulinum toxin formulations and interfere with toxin efficacy in treatment. One of ordinary skill

in the art, therefore, would use column chromatography, as is taught in the above referenced application, to purify botulinum toxin for clinical use and pharmaceutical formulations, and not the methods taught in the references cited by the Examiner in the Office Action, dated June 1, 2004.

3. Furthermore, one of ordinary skill in the art would know that ammonium sulfate precipitated extracts followed by column chromatography could be used to purify all botulinum toxin serotypes. In addition to Melling et al. [Exhibit 2], which provides a detailed description for one of ordinary skill in the art to purify botulinum toxin A, and the above referenced application, which provides detailed teachings on the purification of botulinum toxins A and B, Sacks [Exhibit 3] and Ohishi [Exhibit 4] teach the purification of type E and type F botulinum toxins, respectively, using ammonium sulfate precipitation and ion-exchange chromatography. In addition, Ohishi and Sakaguchi [Exhibit 5] and Ohishi et al. [Exhibit 6] teach the purification of type C, C_2 and D botulinum toxins, again using ammonium sulfate purification followed by ion-exchange chromatography. Ohishi and Sakaguchi, pg. 303, col. 2, 1st full paragraph ("C. botulinum type C and D toxins were purified by essentially the same procedures as those employed for purification of type F progenitor toxin (referencing Ohishi (1974) [Exhibit 5]), except the step of acid precipitation . . ."). Finally Schiavo et al. (1994) also used ammonium sulfate purification followed by ion-exchange chromatography to purify type G toxin. Therefore, one of ordinary skill in the art at the time of filing would know how to purify all botulinum toxins using ammonium sulfate precipitation followed by column chromatography.

4. Furthermore, one of ordinary skill in the art would know from the teachings of the art at the time of filing that the botulinum toxin serotypes, in addition to the similarity in purification methodologies, work identically on the neuromuscular junction and share common structural features. As outlined in Melling et al., all toxin serotypes work on the cholinergic synapses, inhibiting acetylcholine release, and causing muscular paralysis. *See* Melling et al. [Exhibit 3] at pg. 18, col. 2, 1st full paragraph. Finally, all toxin types " . . . although serologically distinct, appear to be structurally very similar to each other; the purified neurotoxins having similar molecular sizes and sharing a common subunit structure." *See* Melling et al., pg. 17, col. 1, 1st and 2nd full paragraphs; pg. 18, Table 1. Specifically, all of the botulinum neurotoxins claimed in the above referenced application bind to receptors at the neuromuscular junction of peripheral cholinergic neurons, are internalized in a similar manner, are zinc metalloproteinases with a similar catalytic site (*see, e.g.*, Schiavo et al. (1994, p. 20213, col. 1, 1st full paragraph), and all claimed botulinum toxins target proteins associated with vesicle release of acetyl-choline (*see Id.*). Finally, the end result of treatment with all of the botulinum toxins claimed in the instant application is a reversible flaccid paralysis. *See Id.* In contrast, another clostridial toxin, tetanus neurotoxin, produces a rigid paralysis (e.g. lockjaw) even though it also shares many features with the botulinum toxins, owing to the extreme specificity of the two different classes of toxins. Therefore, one of skill in the art at the time of filing would recognize the close relationship between the different botulinum toxin serotypes, and recognize

that structurally, biochemically and functionally, the botulinum toxin serotypes all would behave the same in the stabilizing buffer of the above referenced application.

5. Finally, one of ordinary skill in the art would know that the pharmaceutically acceptable agents available for buffering a formulation at between about pH 5.0 and pH 6.0 are within a defined class of compounds. One of ordinary skill in the art would have looked to the 1986 Lachman reference [Exhibit 8], cited in the above referenced application (pg. 14, line 10) and would be able to determine the defined class of compounds when making a pharmaceutically acceptable buffer capable of buffering at between about pH 5.0 to 6.0, including phosphate and succinate buffers, acetate, citrate, aconitate, malate and carbonate as taught in the specification. *See Id.* at pg. 14, lines 10-15. In contrast to the Examiner's assertions that the number of buffer choices would be many, one of ordinary skill in the art would have sufficient guidance as to how to make the claimed formulation simply by the guidance provided by the specification. The specification, therefore, provides sufficient guidance for one of ordinary skill in the art to make and use the claimed invention.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and, further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Date: Apr. 14, 2005

Elizabeth D. Moyer

Elizabeth Moyer, Ph.D.

ELIZABETH D MOYER PHD

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PROFESSIONAL EXPERIENCE

Founder & Chief Executive Officer
M/P Biomedical Consultants LLC (1989 - present)

Provide hands-on project management and strategic planning services to biomedical, biotechnology and pharmaceutical companies to ensure the companies' growth, development and profitability. M/P clients have ranged from multinational corporations such as Pharmacia & Upjohn and Elan Pharmaceuticals, to small or seed money stage companies, and to other consulting groups.

Senior Vice President Product Development and Operations
Kinetek Pharmaceuticals Inc., Vancouver, BC, Canada - (Jun 1999 – May 2000)

Responsible for all phases of Product Development including clinical development and regulatory affairs, as well as managing common service units within Kinetek.

- All responsibilities as outlined for previous position as VP, Product Development
- Drug Development – Identify, evaluate and optimize at least one lead compound from each of three oncology programs, leading to potential preclinical development in 18 months. Establish in vitro and in vivo testing systems, compound/ analog synthesis, and essential analytical systems.
- Operations – Manage central support units for basic research and drug development (physical plant, in vivo and in vitro testing, chemistry, analytical/ formulation, computer/ information systems)

Accomplishments during this period:

- I oversaw three cancer programs that made tremendous strides in developing an operational mode to demonstrate proof of concept of the targets, to assess the efficacy of, and to characterize potential candidate compounds. At least in part because of these efforts, Kinetek was able to raise an additional C$20 million in its second stage of equity financing.
- At the end of my tenure, the Phase I trial for Kinetek's lead clinical candidate, KP-102 was successfully completed, and the program is now poised for Phase II efficacy studies in diabetes.

Vice President Product Development
Kinetek Pharmaceuticals Inc., Vancouver, BC, Canada - (Oct 1997-Jun 1999)

Responsible for coordinating preclinical and clinical development of the company's proprietary technology, for clinical and regulatory affairs, as well as for manufacturing.

- Preclinical development - Oversee pharmacology program, initiate, contract for, and monitor toxicology studies.
- Manufacturing - Initiate, contract for, and oversee manufacturing of drug substance(s) and drug product(s).
- Clinical and Regulatory - Direct the preparation and filing of regulatory documents to initiate clinical trials and to gain marketing approval in North America and Europe. Initiate and oversee clinical trials in North America and Europe. Led meetings with the FDA and HPB.
- Officer of the Company.

Product in clinical trials as a result of my contributions:

- KP-102 - an organo-vanadium compound in-licensed from an academic institution for treatment of Type 2 diabetes in a Phase I clinical trial, accomplished on budget, and in approximately one year.

Director of Preclinical Development
Athena Neurosciences/ Elan Pharmaceuticals, South San Francisco, CA (1989 -Sep 1997)

Responsible for coordinating and providing preclinical and CMC data for regulatory submissions and for managing manufacture of drugs and biologics.

- Basic Research: Established in vivo models of cancer and neurological diseases for efficacy testing of research compounds.
- Product Development: Pharmacokinetics and safety evaluation of drugs and biologics in various therapeutic classes, primarily for central and peripheral nervous system diseases and cancer therapeutics. Identified and evaluated contract facilities and managed contracts for GLP pharmacology, toxicology testing. Formulation of small molecule parenterals and proteins and inventor of a patented formulation for an injectable protein. Member of various Project Teams.
- Clinical & Regulatory Affairs: Rewrote, reformatted, and reassembled in-licensed product documentation for FDA submission. Provided pharmacology, toxicology, pharmacokinetics, regulatory, quality assurance, and manufacturing information for, and/or generate drafts of Orphan Drug Applications, IND's, and marketing applications for the United States, Canada, and United Kingdom. Participated in FDA contacts and meetings, and helped negotiate key issues leading to market approval. Assisted in clinical trial protocol development/ writing Investigator's Brochures and negotiated key issues leading to market approval.
- Manufacturing: Identified and evaluated contract facilities and managed contracts for GMP biologics (bacterial products and recombinant proteins) manufacturing, fill and finishing facilities. Member, validation committees for biologics and drugs, assisted in development of Master Validation plan for Biologic. Wrote IQ/OQ/PQ for process equipment, validation protocols for analytical methods including bioassay, and SOP's related to facility, process, analytical equipment, and analytical methods. Designed stability protocols, established product release criteria. Assisted in design and validation of aseptic manufacturing facility. Trained in-house research personnel in GMP and GLP.
- Product Licensing: Evaluated pharmaceutical literature for competing products. Made recommendations regarding in-licensing and out-licensing.
- Marketing: Provided analysis of technical materials in terms suitable for marketing personnel. Reviewed marketing materials, and participated in market launch of two products.
- Other: Designed and supervised USDA-certified small animal facility for small animals (including transgenic mice). Hired, trained, and supervised animal care personnel responsible for facility. Also, trained and supervised company personnel in basic in vivo techniques.
- Chairperson, Institutional Animal Use and Care Committee. Insured protocols, SOP's and documentation involving use of animals met USDA and NIH guidelines.

Key participation in clinical development, approval and/or postmarketing of the following products:

- DIASTAT (DIAZEPAM Gel) - Rectal gel formulation for treatment of acute repetitive seizures (anitconvulsant), primarily pediatric use, in various dosage forms and strengths
- TIZANIDINE HYDROCHLORIDE (ZANAFLEX) – Tablet, Oral, for treatment of spasticity associated with spinal cord injury or multiple sclerosis. In-licensed from Novartis.
- PERMAX, Tablet, Oral - a dopamine agonist indicated as adjunct therapy for the treatment of Parkinson's disease, co-marketing agreement with Eli Lilly.
- CARBATROL – oral granulation formulation of carbamazepine, in-licensed from Shire, NDA approved 1998, re-licensed to Shire

- Athena line of oral table generic neurological drugs, including baclofen (ATROFEN), carbamazepine (ATRETOL).
- NEUROBLOC (Botulinum Toxin Type B), an injectable protein drug for neck-muscle spasms, recently approved in the US and EU for marketing, Canadian application still under review.
- TYSABRI (ANTEGREN), a multiple-sclerosis monoclonal antibody drug, recently approved in the US for multiple sclerosis and in Phase III trials for irritable bowel syndrome.

Senior Research Scientist
Pharmacia (Kabi Pharmacia) (1984 - 1989)

Responsible for direction and conduct of basic research.

- Basic Research: Conducted experiments to evaluate efficacy and metabolism of drug candidates in normal, immunostimulated, hemorrhage and trauma models. Performed in vivo contract research with blood substitute and growth factor products. Managed contract research in a surgical/trauma research laboratory. Evaluated scientific merit of internal and external research proposals.
- Clinical & Regulatory Affairs: Prepared two New Drug Applications, obtaining $2M bonus from marketing partner company. This involved integrating preclinical, clinical, and manufacturing data from company (Sweden and US) and its partners, writing appropriate summaries, and assembling all documentation. Generated draft labeling for these new products including package inserts and container label mockups appropriate to the collaborate agreements.
- Other: Prepared statistical evaluation and graphing of experimental and manufacturing stability data for presentation at national research meetings, business presentations, and to the FDA. Designed and supervised USDA-certifiable animal facility. Established and maintained health monitoring system to insure pathogen-free animal facility.

Products approved or in clinical trials as a result of my contributions:

- 30% INTRALIPID (SOYBEAN OIL EMULSION, INTRAVENOUS) – parenteral formulation for nutritional support: FDA and European marketing approvals.
- NEOPHAM T (PEDIATRIC AMINO ACIDS, INTRAVENOUS) – parenteral formulation for nutritional support (NDA withdrawn pending reformulation in plastic delivery system).
- Novel triglyceride oil emulsion (still in clinical investigation).

Research Scientist
Miles Laboratory (Cutter Group) and Pharmacia (Kabi Pharmacia), Inc. (1982 - 1984)

Responsible for direction and conduct of basic research.

- Basic Research: Conducted mouse, rat, rabbit and dog experiments to evaluate efficacy, pharmacokinetics, metabolism and safety of intravenous products, nutritional and biological. Assisted in GLP safety studies, including long term (28 and 90 day) infusion studies. Developed rat metabolic stress models for evaluation of acute clearance, and analytical methods as necessary.
- Project Management: Associate product manager for novel parenteral designed for use in sepsis. In this role, responsible for project planning and coordination of biological research, analytical, experimental production, and toxicology groups.

Résumé: Elizabeth D Moyer PhD

EDUCATION

B.A. Chemistry and Biology, 1974, Randolph Macon Woman's College, Lynchburg, VA. Awarded a Given Distinguished Scholarship.

Ph.D. Biochemistry (Clinical chemistry predoctoral program, AACC accredited), 1978, State University of New York at Buffalo, Buffalo, NY.

- Studied protein and energy metabolism in septic trauma patients. Performed statistical evaluation of large clinical data bases using parametric and nonparametric methods. Evaluated clinical records and standard clinical tests for liver, renal, cardiac function for inclusion / exclusion of patients from clinical categories.

Postdoctoral fellowship (1978 - 1980): Departments of Biochemistry and Surgery, State University of New York at Buffalo, Buffalo, NY.

- Conducted interdisciplinary research involving metabolic and physiologic alterations induced by sepsis, trauma, cancer and cirrhosis in critical care patients, and the influence of malnutrition and parenteral nutrition on these changes. Studied plasma protein alterations associated with severe metabolic stress, and the implications of these changes to wound healing and recovery.
- Acting head of trauma research biochemistry laboratory: responsible for supervision and management of biochemical analytical laboratory [three technicians (one Ph.D.), two computer programmers, a graduate student, and a part-time clerical assistant].

Postdoctoral fellowship (1980 - 1982): Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, MD. NSF training fellowship. Amino acid and alpha-keto acid metabolism in laboratory animals and man (trauma, cancer, and liver failure).

- Assisted in the design and management of clinical trials of intravenous products in cancer, cirrhotic and surgical trauma patients. Evaluated clinical histories and clinical laboratory data. Set up and used rat hindlimb perfusion model in rats for evaluation of peripheral clearance.

PROFESSIONAL TRAINING / COURSES

Various, including:

- Harvard Law School – Program on Negotiation for Senior Executives, March 2000
- Parenteral Drug Association – Formulation of Solid Dosage Forms, June 1999
- CBER/NIH - Gene Therapy Conference, July 1996
- Parenteral Drug Association – Assay / Bioassay Validation, June 1996
- Interactive Workshop on ICH Guidelines, May 1996
- FDA Workshop – Policy Issues in the Development & Manufacture of Biopharmaceuticals, May 1995
- Center for Professional Advancement – Stability of Protein Drugs, Biologics and Devices, March 1992

PATENTS

Stable Liquid Formulations of Botulinum Toxin –US Pat App No 09/393,590

PUBLICATIONS AND REFERENCES

List available on request.

Eye (1988) 2, 16–23

Clostridium Botulinum Toxins: Nature and Preparation for Clinical Use

J. MELLING, P. HAMBLETON and C. C. SHONE
Salisbury

Summary

***C. botulinum* neurotoxins are acutely toxic materials and act by inhibiting release of the neurotransmitter acetylcholine. The specific nature of this inhibition is discussed and the preparation and purification of Type A toxin specifically for clinical use is described.**

There have been few drugs whose mode and site of action and detailed pharmacology have been so well understood prior to their clinical application as Type A botulinum toxin. Indeed, the nature of this family of neuroparalytic agents as the most acutely potent microbial toxins and the cause of the food-borne illness botulism has made regulatory authorities particularly cautious in approving their clinical application. Nevertheless the use of the toxin particularly to treat a variety of dystonic conditions represents a most elegant, precise and safe treatment which reflects considerable credit on the pioneering work of Scott[1] who first instituted the use of the toxin for human therapy.

The therapeutic, as well as the prophylactic (vaccine) use of microbial products is, of course, well established and as well as native products, especially antibiotics, also includes bacterial asparaginases used to treat acute lymphoid and lymphoblastic leukaemia in children.[2] Increasingly we are also seeing human gene products such as insulin, growth hormone and interferons produced in micro-organisms and used for human therapy. It is virtually certain that many other potent human regulatory proteins will also become available by this route.

Thus when it comes to pharmaceutical production *C. botulinum* toxin can be viewed as one of the first of a group of high activity protein products prepared from cultures of micro-organisms and the problems and solutions in handling such materials have general applications.

The purpose of this article is to review the nature of *C. botulinum* toxins and to describe the procedures involved in the production, purification and presentation of the type A toxin for clinical use.

The Nature of the Toxins

Structure

Complex toxins. There are eight serologically distinct toxins (A, B, C_1, C_2, D, E, F and G) produced by *C. botulinum* that are among the most powerful neuroparalytic agents known. The toxins so far studied all appear to occur both naturally and in *in vitro* culture complexed with non-toxic proteins which may co-purify with the toxin moiety (Fig. 1). Of these complexes the smallest, the M or medium complexes, comprise the neurotoxin moiety (ca. 150,000 daltons) associated with a similar sized non-toxic protein of undetermined biological activity and are the only forms found for the types C[3], E[4,5] and F[6] and

From Vaccine Research and Production Laboratory, PHLS, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire.

Correspondence to: J. Melling, CAMR, Porton Down, Salisbury, Wilts SP4 0JG, UK.

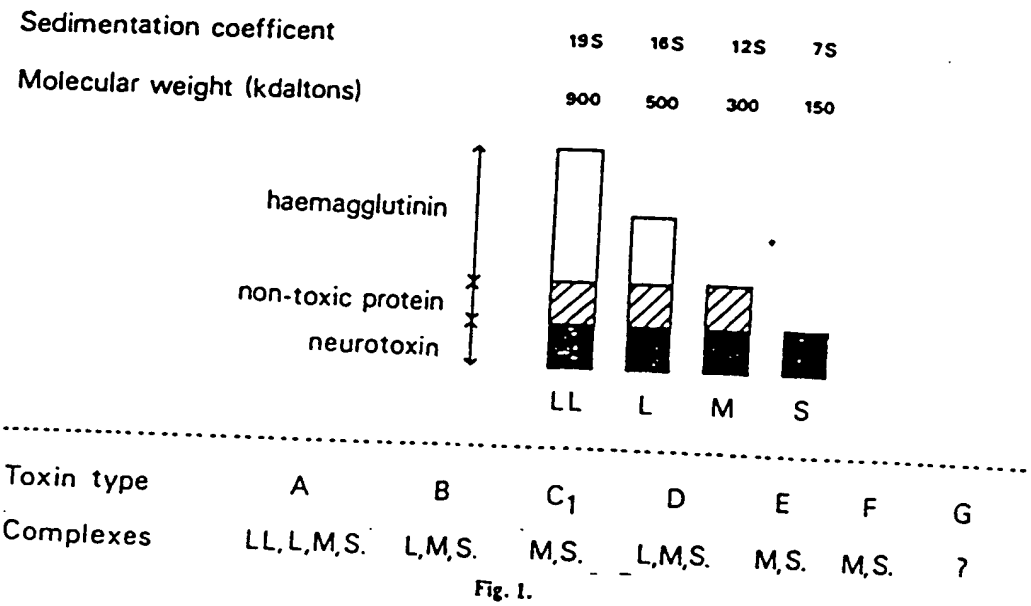

Fig. 1.

haemagglutinin-negative strains of type D[7]. Types A[8], B[9] and haemagglutinin—positive D[7] also occur as trimolecular or L (large) complexes comprising the M complex in association with a non-toxic protein having haemagglutinin activity. The L complexes (Ca. 500,000 daltons) are the largest complex states of these toxins with the exception of type A toxin which also occurs as a complex of about 900,000 daltons. This latter complex, which is thought to consist of dimers of the L complex, is the molecular size of the type A toxin in the crystalline state.

Neurotoxins. The various *C. botulinum* neurotoxins, although serologically distinct, appear to be structurally very similar to each other: the purified neurotoxins having similar molecular sizes and sharing a common subunit structure (Table II). These single chain progenitor toxins may be cleaved by proteolytic enzymes to give biologically active dichain toxins (Fig. 2).

The dichain toxins consist of one large (heavy, H) and one small (light, L) subunit linked by at least one disulphide bridge: the H subunits are about double the molecular size of the L subunits[4,6,10,19] (Table I).

Fig. 2.

The amino acid composition of several toxin types (A, B, C, D, E and F) have been evaluated[16,18,20-22] and similarities are apparent. In particular all have high contents of hydrophobic amino acids residues which may be relevant to the membrane binding/internalisation of the toxin as discussed later. The similarities in amino acid composition together with their shared pharmacological action imply that, despite their lack of antigenic cross-reactivity, there may well be regions of structural homology within the neurotoxins.

Neurotoxin subunits. Reduction of the disulphide link(s) between H and L subunits generally leads to a loss of toxicity and a reduction in the solubility of the toxin. The subunits of reduced neurotoxin remain associated by covalent forces but these can be dissociated and solubilised by urea and subsequently separated chromatographically.[16-18,23-25]

Purified H and L subunits of the neurotoxins are of greatly reduced specific toxicity (<0.5 per cent) compared to the intact toxin; the residual toxicity probably being due to trace contamination with intact toxin.[16] Separated neurotoxin subunits appear to maintain their structural integrity and can be recombined into intact neurotoxin by mixing equimolar portions of each subunit, in reducing conditions, and slowly removing the

Table I *Molecular size and subunit structure of* C. botulinum *neurotoxins*

Toxin type	Molecular size (k daltons)			Reference
	Holotoxin	H subunit	L subunit	
A	145	95–97	55	10, 11
B	155–170	101–104	51–59	12–14
C_1	141–144	92–98	52–53	15–17
D	140	85	55	18
E	147–150	102	50	4
F	155	105	56	6, 19
G	Ca.150?	Not yet chracterised		

reducing agent. Some 30 per cent of the potential toxicities of reconstituted types A[23] and B[24] toxins were regained and up to 70 per cent for type C, toxin.[16]

Toxicity. When administered by the parenteral route the non-toxic proteins of the complexes appear not to play a role in toxicity, indeed, the highest specific toxicities are observed with the purified neurotoxin component (Table II). For example, the purified 145,000 dalton type A neurotoxin (S-form) has a specific toxicity about 3 fold higher than that of its L complex. In contrast, the complex toxins are more toxic than purified neurotoxins when administered orally (Table II). This difference is even more pronounced with the type B toxin where oral toxicities of the S, M and L forms are in the ratio of 1/2/1,600 respectively.[26] Differences in the oral toxicities of toxins from two different type B strains of *C. botulinum* were attributed entirely to differences in the properties of the non-toxic proteins of the respective complex toxins. The enhanced oral toxicity of the complex toxins probably reflects the ability of the non-toxic proteins to protect the neurotoxin moiety from the hostile environment of the digestive tract prior to its uptake into the blood and lymphatic systems.[27,28]

Table II *Enteral and parenteral toxicities of different molecular forms of* C. botulinum *Type A toxin*

Molecular form	Intraperitoneal route mouse LD_{50} mg protein	Oral Route mouse LD_{50} mg protein
LL	3.8×10^{7}	3.2×10^{5}
L	4.8×10^{7}	2.2×10^{6}
M	8.0×10^{7}	2.2×10^{8}
S	1.6×10^{8}	3.7×10^{8}

Data calculated from Sakaguchi *et al.*[26]

Mode of Action

The botulinum neurotoxins act primarily on the peripheral cholinergic synapses where, by inhibiting the release of the neurotransmitter acetylcholine, they cause the widespread muscular paralysis characteristic of the fatal syndrome botulism. Studies with isolated neuromuscular junction preparations show that the action of the toxin is not mediated merely by its binding to the nerve membrane; since binding is rapid, whereas paralysis has a slower onset.[30] Furthermore, in contrast to the rapid binding, stimulation of the nerve is required to induce paralysis which is temperature sensitive and requires the presence of calcium ions in the fluid bathing the neuromuscular junction.[31–33]

It is now believed that at least three steps are involved in the inhibitory action of botulinum toxins. Firstly, there is a binding step, whereby the toxin attaches rapidly and avidly to the presynaptic nerve membrane. Secondly, an internalisation step in which toxin crosses the presynaptic membrane and a final step, or steps, whereby toxin inhibits the release of the neurotransmitter substance, acetylcholine.

Toxin binding. Selective, saturable binding of botulinum neurotoxins to presynaptic nerve membranes of both peripheral and central nerves has been shown using labelled toxin molecules.[34–36] Neurotoxin visualised with ferritin-labelled antibody was observed to bind in discrete patches of varying size rather than uniformly over the nerve surface.[36]

Studies using synaptosomes from rat or mouse brain have shown that the binding of

radio-labelled t
of unlabelled t
number of binc
types of accep
involved: a smal
rotoxin with hi
larger pool of a
lower affinity (K
assumes that e
one binding (ac
likely that the to
high affinity site
affinity ones.

Table III *Bindin*
synaptosomal acce

Toxin type	I.
A	0
B	0.
C	0
D	71

Although the
macological pro
ulinum neurotox
have similar moc
that the synapto
fer from some
indeed, be at le
mal acceptors, or
toxins and one
there is little or n
A and B or B ar
somes whereas T
pete for the
Similarly, bindir
pletely inhibited
mon acceptor co
are similar to the
or B toxins is ne
The role of subu
those botulinum
the H subunit a
site which binds
For both A a
unlabelled H-su
radio-labelled to
without effect. Si
is inhibited by T

that the normal intracellular Ca^{2+} levels are insufficient to promote release.

Similar conclusions are indicated by studies with substances such as tetraethylammonium[43] and 4-aminopyridine[44–46] which, in prolonging the depolarisation/repolarisation cycle of the nerve, allow higher concentrations of Ca^{2+} to build up in the nerve terminal. In these conditions the end plate potentials of mildly botulinum-poisoned neuromuscular junction preparations can be restored to normal.

Many key aspects of the action of the botulinum neurotoxins still await clarification, including the appearance of minature end plate potentials shortly after the onset of poisoning and particularly the long duration of the toxic effects. One possibility is that the toxin evokes a permanent change in an enzyme, or other vital system, having a long half life.[47–50] Indeed it has been proposed that, in common with some other microbial toxins, the toxin itself may be an enzyme: the protracted action and high specificity of the toxin appears to support this view.

Preparation of Type A Toxin for Clinical Use

For a product to be acceptable and licensable for clinical use appropriate standards of quality, safety and efficacy must be achieved. Thus material must be reproducible in its composition and potency from batch to batch and stable during a particular period under defined and achievable storage conditions. Safety and efficacy need to be assessed both by animal experimentation and in human trials and to be verifiable on subsequent batches by the application of relevant quality control tests. All of the above requires the application of Good Manufacturing Practice (GMP) techniques. GMP builds on laboratory biochemistry and microbiology and translates a Research and Development process into an effective pharmaceutical operation. The importance of standardising conditions for production and purification is clearly apparent given the effect of proteolytic 'nicking' on toxic activity and the further susceptibility of the protein toxin to inactivation by proteolytic enzymes.

Culture. Cultures of *C. botulinum* from a verified seed stock are grown up and inoculated into a 30 l fermenter operated under anaerobic conditions and toxin production and other culture parameters are monitored (Fig. 3). When the maximum yield of toxin has been attained (2×10^8 mouse LD_{50}/ml) usually after 72 hours the toxin is harvested by centrifugation after acidifying the culture. In this form the toxin can be stored prior to purification. Cultures are extensively checked for toxin activitiy, identity and absence of contamination.

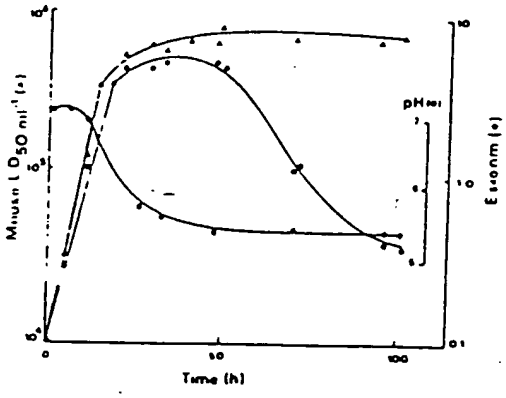

Fig. 3.

Purification. The precipitated crude toxin is re-dissolved and purified by a series of procedures involving ammonium sulphate precipitation and ion-exchange chromatography (Table IV). During these procedures the process is monitored for the absence of contamination with extraneous micro-organisms as well as the toxicity and protein content of the extracts.

Formulation and Freeze Drying. The potency of the purified toxin is assessed and an appropriate quantity of the purified toxin solution is added to a diluent containing lactose and human serum albumin. The diluent is designed to provide protection for the toxin during freeze-drying and to act as a bulking agent for the freeze dried product. Prior to freeze drying, the diluted toxin is dispensed into vials and at the completion of the drying process vials are checked for integrity, sterility, moisture content and potency.

Clinical Use and Dosage

C. botulinum toxin has now been used to treat both experimentally and routinely a range of

Table IV *Pur*

Stage procedure
1 Whole culture
2 Precipitation … H_2SO_4
3 Extraction wit pH 6.0
4 Ribonuclease
5 Precipitation … of ammonium
6 DEAE-Sephac
7 DEAE-Sephac graphy at pH …
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Table IV *Purification of C. botulinum Type A Hemagglutinin-neurotoxin Complex*[46]

Stage procedure	Protein (mg)	Toxicity: Total mouse LD_{50}	Toxicity: MLD_{50}/mg protein	Recovery %: Stage	Recovery %: Overall
1 Whole culture (20.1)	—	10^{10}	—	100	100
2 Precipitation at pH 3.5, and adjusted with 3N H_2SO_4	4.600	7.5×10^{9}	1.6×10^{6}	75	75
3 Extraction with 0.2M phosphate buffer at pH 6.0	1.300	6.8×10^{9}	5.2×10^{6}	91	68
4 Ribonuclease treatment (100 ug/ml, 34°, 3 h)	1.300	6.8×10^{9}	5.2×10^{6}	100	68
5 Precipitation at 60 per cent saturation (at 25°) of ammonium sulphate	—	6.5×10^{9}	—	96	65
6 DEAE-Sephadex A50 batch preabsorption	860	5.0×10^{9}	5.8×10^{6}	77	50
7 DEAE-Sephacel ion-exchange chromatography at pH 5.5	195	4.8×10^{9}	2.5×10^{7}	96	48
8 Precipitation at 60 per cent saturation (at 25°) if ammonium sulphate	195	4.8×10^{9}	2.5×10^{7}	100	48

conditions where deliberate paralysis of a particular muscle may be of benefit. The conditions include strabismus,[1] blepharospasm,[49] lateral rectus paresis,[50] hemifacialspasm,[51] Meige syndrome,[52] facial synkinesis after facial nerve palsy and spasmodic torticollis.[53]

Clearly the muscle sizes, and most critically the number of cholinergic synapses, vary considerably among these conditions as it is clear does the dose of toxin required for effective action. It is likely that for the effective treatment of patients and the convenience of clinicians toxin will need to be made available in a range of doses appropriate to the particular condition and even for one condition individual patient responses may dictate varying doses. Accordingly we decided to present the toxin which has been used for blepharospasm and strabismus treatment as a weight of active protein determined by mouse potency; the use of units of activity based on human physiological activity may follow as more clinical data become available.

The stability of the toxin preparation over time is clearly critical to building up an effective clinical data base and we are able to confirm that our freeze-dried toxin retains stability even when kept at 4° C for not less than 3 years. Thus the potency of the preparation is a constant factor even when used in different clinics, countries and over a long time period and should enable a consistent dose regime to be established for a range of dystonia conditions.

As the data presented above show, not only is the mode and site of action of the toxin highly specific but it also possesses a further important characteristic which is its avidity for the site of action. Taken together these characteristics allow local injection into a muscle with little or no general dissemination. It will eventually be interesting to investigate the clinical use of the pure 150,000 MW neurotoxin as this molecule which does not have the haemagglutinin moiety of the complex toxin may give even better precision and reproducibility of action.

Further development may be required if there is evidence of production of antibody by patients receiving toxin. Should this become a problem it would be necessary to make available other of the serologically distinct toxin Types B through G and the information currently being pursued on the structure and activity of these other toxins should prove invaluable in assessing their suitability for clinical use.

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Clostridium botulinum Type E Toxin: Effect of pH and Method of Purification on Molecular Weight

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The toxin of *Clostridium botulinum* type E was isolated from intact cells and from toxic culture filtrates by column chromatography at three pH values, 4.5, 5.3, and 6.0. At pH 6.0 and 5.3, the isolated toxin was in a form with a molecular weight (MW) of 86,000. This toxin was homogeneous on polyacrylamide gel electrophoresis and gel filtration and had an optical density ratio, 280 nm/260 nm, greater than 2.0. It did not dissociate at higher pH levels, but was dissociated into nontoxic components of approximately 12,000 MW when reduced and alkylated in the presence of 6 M guanidine hydrochloride. At pH 4.5, smaller amounts of an impure toxic moiety with a MW of 12,000 were found. After storage for 6 months, the 86,000-MW moiety had lost 60% of its lethality. Gel filtration revealed that the bulk of the toxicity was associated with a component having a MW of 150,000. Toxic components with MW of 12,000 and over 200,000 were also found. The toxin appears to polymerize or aggregate when in a pure form, so that most, if not all, of the MW previously reported for the toxin may belong to different polymers of a monomer with a MW of 12,000 or less. Treatment of the 86,000-MW toxin with trypsin resulted in an 18- to 128-fold increase in lethality, but no detectable change in MW.

Estimates for the molecular weights (MW) of toxic proteins isolated from *Clostridium botulinum* type E have ranged from 5,000 (3) to near 900,000 (16). Most attempts to isolate the toxic moiety have involved the use of selective precipitation, gel filtration, and ion-exchange chromatography, but there have been important differences in the techniques employed. Gerwing et al. (5, 6) purified toxic proteins from cell-fee culture filtrates by gel filtration and ion-exchange chromatography of solutions buffered at pH 4.5. They estimated the MW of their preparation to be 12,000 to 18,000. Emodi and Lechowich (3) precipitated toxin from the culture medium with ammonium sulfate after first spinning out the cells. Gel filtration on Sephadex G-200 suggested that the toxic moieties had MW of 5,000 and 9,000. Kitamura (10) has conducted ultracentrifugal analyses of a toxin prepared by the method of Emodi and Lechowich and found evidence for a high-molecular-weight (HMW) toxin in the preparation. These methods have been classified as preparations of released toxin (10).

An alternative approach has been to extract retained botulinum toxin from whole cells, using 1 M sodium acetate or 0.2 M phosphate buffer (12). Gel filtration and ion-exchange chromatography at pH 6.0 yielded a product with a MW estimated by ultracentrifugation to be 350,000 (13). This toxin was homogeneous at pH 6.0, but at pH 8.0 it could be separated into toxic and nontoxic components, labeled E_α and E_β, respectively, each with a MW of 150,000.

The present study is an attempt to clarify the roles of pH and of released or retained origin in determining molecular size of the toxin obtained, and to study further the dissociation of the toxin.

MATERIALS AND METHODS

Organisms and culture methods. Samples of *C. botulinum* type E, VH strain, (obtained from E. J. Schantz, Fort Detrick, Frederick, Md.) were stored in a dry-ice chest. Organisms were grown in a liquid medium containing 1.5% Casitone (Difco Laboratories, Detroit, Mich.), 0.25% yeast extract (Baltimore Biological Laboratory, Cockeysville, Md.), 1% glucose, 0.05% $CaCl_2$, 0.1% K_2HPO_4, and 0.37% cysteine hydrochloride. The bacteria were incubated at 30 C in flasks containing 2 to 8 liters of medium.

Purification of retained toxin from CEX. The general scheme for purification of retained toxin is given in Fig. 1. The cells were harvested by continuous centrifugation at room temperature in a DeLaval Gyrotester (The DeLaval Separator Company, Poughkeepsie, N.Y.). The cells were washed twice by

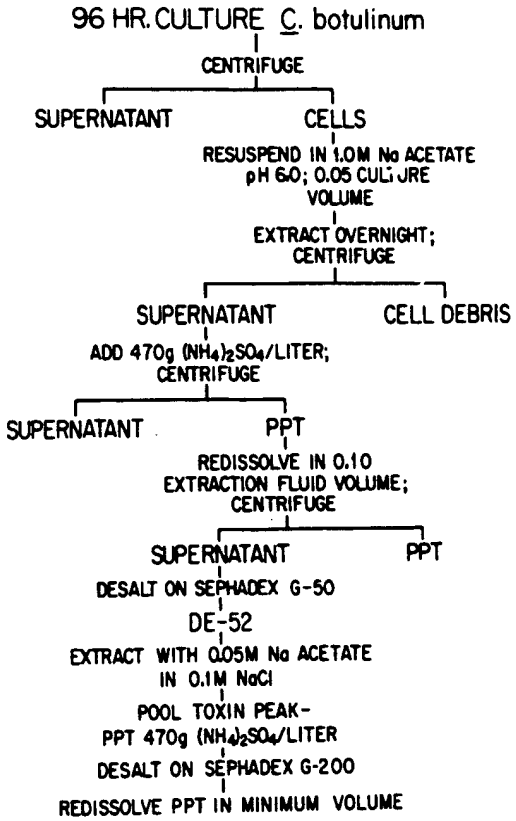

FIG. 1. *Flow diagram for the purification of C. botulinum type E toxin. This scheme was used with minor modifications throughout the range of conditions employed.*

suspending them in distilled water and centrifuging them in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with a GSA rotor at 10,000 rpm (13,600 × *g*) for 15 min. The cells were extracted with a 0.05-culture volume of 1.0 M acetate buffer (pH 6.0) at 4 C for 24 h. Toxin was precipitated from the cell extract (CEX) by the addition of ammonium sulfate (special enzyme grade, Mann Research Laboratories, New York, N.Y.) to 70% saturation (470 g/liter). The precipitate was allowed to form for 24 h. at 4 C and was then collected by centrifugation in a Sorvall RC2-B centrifuge at 13,600 × *g* for 15 min. The precipitate was suspended in 0.1 volume of 0.05 N acetate buffer (pH 6.0). Insoluble material was removed by centrifugation.

The CEX was desalted, and low-molecular-weight (LMW) contaminants were removed by gel filtration with Sephadex G-50 columns (2.5 by 40 cm). Flow was regulated at 2 ml/cm²/h with a Holter bilateral roller pump (The Holter Company, Bridgeport, Pa.). Samples (5 ml) of the outflow from the columns were collected in a Gilson model VL linear fractionator (Gilson Medical Electronics, Middleton, Wisc.). Elution with 0.05 N acetate buffer (pH 6.0) was followed by optical density (OD) measurements at 260 and 280 nm in a Beckman model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

The contents of the tubes containing the highest toxic activity concentrations were pooled. The pooled toxin solution was applied to columns (2.5 by 15 cm) packed with Whatman DE-52. The toxin was eluted by a stepwise gradient of NaCl in the same acetate buffer. NaCl was added in 0.1-M increments to a final concentration of 1.0 M NaCl.

Eluates with the highest toxicity were pooled. The toxin was precipitated by the addition of ammonium sulfate to 70% saturation (470 g/liter). The precipitate was allowed to form for 24 h at 4 C and was then collected by centrifugation in a Sorvall RC2-B centrifuge with SE-12 rotor at 10,000 rpm (10,300 × *g*) for 15 min. The precipitate was suspended in 0.1 volume of acetate buffer (0.05 N, pH 6.0), and the insoluble material was removed by a second centrifugation.

The final product was obtained by gel filtration on columns (2.5 by 97.5 cm) of Sephadex G-200. The toxin was filtered through sterile membrane filters (Millipore Corp.) and stored at 4 C. Samples were removed periodically to determine toxin stability.

Purification was carried out by the same general procedure, but by substituting the following buffers for 0.05 N acetate (pH 6.0): 0.05 N acetate (pH 4.5) and 0.05 N acetate (pH 5.3). One series was run with 0.025 N acetate buffer of pH 4.5, which also contained 0.05 M guanidine hydrochloride (ultrapure, Mann Research Laboratories). Also, for one series, the ammonium sulfate precipitations were omitted. Instead, the CEX was concentrated by adding Sephadex G-25 (coarse; 20 g/100 ml). The Sephadex was allowed to swell for 1 h. The concentrated CEX was recovered by vacuum filtration through a Buchner funnel lined with 400-mesh nylon netting. Sephadex G-25 (coarse) was added again, and the procedure was repeated. In this series, smaller columns (1 by 30 cm) of Sephadex G-200 were used.

Purification of released toxin from culture filtrate. The procedures for purification of toxin from the culture filtrate were similar to that just described for CEX, except that the starting material was the supernatant remaining after centrifugation of the cells. Purifications from the culture filtrate were carried out at pH values of 4.5, 5.3, and 6.0. The purifications at pH 4.5 and 6.0 were done in the presence of 0.05 N guanidine hydrochloride within the buffers.

Trypsinization. Samples of toxin were incubated with bovine pancreatic trypsin (EC 3.4.4.4; recrystalized two times, Sigma Chemical Co., St. Louis, Mo.) in a final concentration of 0.01% for 2 to 6 h in a 37 C water bath. Action of the enzyme was halted by the addition of soybean trypsin inhibitor type I-S (Sigma, crystallized two times), or by adjustment of the pH to 4.0.

Polyacrylamide gel electrophoresis was carried out by modification of the Hjerten method (9). Electrophoresis was done at pH 8.0 in 0.05 tris(hydroxymethyl)aminomethane-phosphate buffer and at pH 4.0 in 0.07 N glycine-acetic acid. Samples were diluted with 30% sucrose solution to give protein concentrations of approximately 0.5 mg/ml and were layered

over gels in Pyrex tubes (5 by 70 mm). A loading current of 2 mA per tube was applied for 15 min and then increased to 5 mA per tube for the remainder of the run (2 to 3 h). The gels were stained with 1% Buffalo black in 7% acetic acid.

Mouse assay. Toxicity determinations were made on toxin samples diluted with a 0.85% NaCl solution containing 0.1% gelatin. Lethality was determined by intraperitoneal injections of 0.5-ml serial twofold dilutions into female white mice (17- to 20-g) using two to six mice per dilution. The mean lethal dose (LD_{50}) was calculated by the Reed and Muench method (14).

Characterization of the toxin molecule. MW of toxin preparations were determined by gel filtration on a column (2.5 by 100 cm) of Sephadex G-200 (1). The eluting buffer was 0.05 N K_2HPO_4-KH_2PO_4, pH 6.0. The column was calibrated with the following proteins: lysozyme (Sigma, grade I), MW = 14,300; bovine pancreatic trypsin (Sigma, crystallized two times), MW = 22,000; egg albumin (Sigma, grade V), MW = 45,000; bovine albumin (Sigma, fraction V), MW = 68,000; bovine gamma globulin (Mann Research Lab., fraction II), MW = 156,000; and bovine thyroglobulin (Sigma, type I), MW = 600,000. The MW quoted above are commonly accepted literature values.

Each protein (10 to 20 mg) was dissolved in 2 ml of eluting buffer and applied to the column. Flow was regulated at 2 ml per cm^2 per h by a Holter bilateral roller pump. Samples (2 ml) were collected in a Gilson fractionator. Elution of protein was monitored by measuring OD at 280 nm with a Beckman model DB spectrophotometer. Each protein was run at least four times.

Gel filtration in the presence of guanidine hydrochloride was carried out by using a modification of the method of Fish et al. (4). Protein samples were dissolved in 0.5 ml of 6 M guanidine hydrochloride, pH 6.5, (ultrapure, Mann Research Laboratories) and applied to columns (1 by 30 cm) of Sephadex G-200. They were eluted with the same solvent. Reduced and alkylated samples were prepared by dissolution in 0.5 ml of 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol (pH 8.6) for 4 h and then by adding iodoacetic acid to 0.25 M and lowering the pH to 6.5 before applying the samples to the column.

RESULTS

Preparation of retained toxin at pH 6.0. After 96 h of culture, approximately 90% of the recoverable toxin was intracellular and extractable with sodium acetate solution. The toxic material was concentrated by ammonium sulfate precipitation rather than by lyophilization, because we found that the latter procedure greatly reduced the lethality of the preparation.

Gel filtration of the concentrated CEX is shown in Fig. 2. The principal peak at the void volume (V_o) of the column contained the toxin. However, it also contained a large percentage of ribonucleic acid (RNA), as shown by the fact that the extinction at 280 nm is less than that at 260 nm, and by a positive Bial reaction for pentose. The RNA was separated from the toxin by ion-exchange chromatography (Fig. 3). The first peak contained up to 160,000 LD_{50}/ml. This is the only peak for which the extinction at 280 nm exceeds that at 260 nm. The latter peaks, which contained the RNA, all had less than 8,000 LD_{50}/ml. Only those samples with OD_{280}/OD_{260} ratios greater than 1.9 were found to be free of RNA by Bial reaction.

Kitamura et al. (11) found that their toxic eluate at pH 6.0 from DE-52 was homogeneous on electrophoresis at pH 6.0, but had two components at pH 8.0. They used ion-exchange chromatography at pH 8.0 to separate these components. We were unable to confirm this finding: polyacrylamide gel electrophoresis at pH 8.0 showed that the central portion of our pH 6.0 eluate from DE-52 contained only one component (Fig. 4). The material was also homogeneous on ion-exchange chromatography at pH 8.0.

The toxin was then chromatographed on a column of Sephadex G-200 to obtain the final

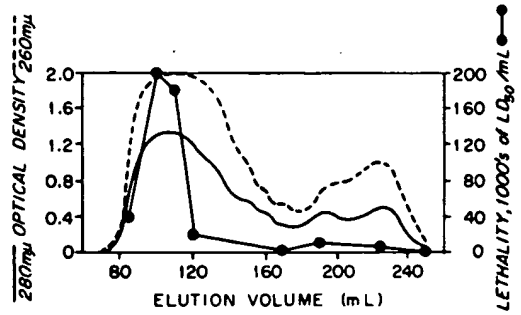

FIG. 2. *Gel filtration of concentrated CEX on a Sephadex G-50 column (2.5 by 40 cm) at pH 6.0.*

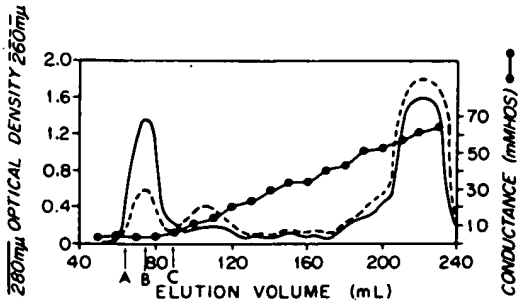

FIG. 3. *Chromatography of CEX on a Whatman DE-52 column (2.5 by 15 cm) at pH 6.0 with stepwise addition of NaCl. (A, B, and C) Points where samples were taken for electrophoresis. Only the first peak contained significant toxicity.*

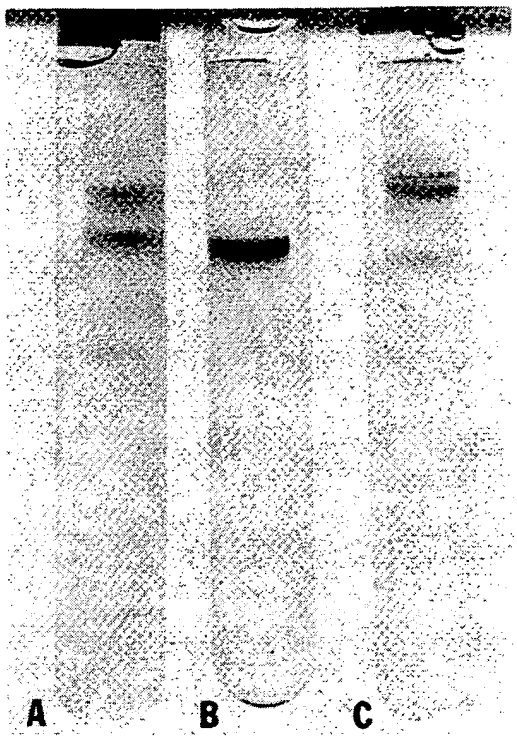

FIG. 4. *Polyacrylamide gel electrophoresis of a pH 6.0 eluate from Whatman DE-52 at elution volumes of 65 (A), 75 (B), and 88 (C) ml.*

product (Fig. 5). There was one major peak at approximately 1.8 × V_o. The leading and trailing edges of this peak were not symmetrical, indicating that they may have contained more than one component. The central area of the peak closely approximated a normal curve and contained the toxin in a highly purified form (Fig. 6). There was also a small nontoxic fraction that was eluted considerably later. Rechromatography of the toxin on Sephadex G-200 gave a single symmetrical peak.

Preparation of retained toxin at pH 4.5. Lowering the pH from 6.0 to 4.5 produced a considerable change in the behavior of the toxic material on Sephadex G-50 (Fig. 7). The first peak, at V_o, was smaller and less toxic, and the OD at 280 nm exceeded that at 260 nm. Whereas at pH 6.0 there had been a small peak at around 2.3 × V_o, there was a much larger, more toxic fraction at this point. The fractions collected at this point were turbid. Upon standing overnight at 4 C, a precipitate formed. This was collected by centrifugation and was suspended in phosphate buffer (pH 6.0). It contained low levels of toxicity (2,000 LD_{50}/ml to 4,000 LD_{50}/ml); the toxicity of the supernatant fluid remained high (200,000 LD_{50}/ml).

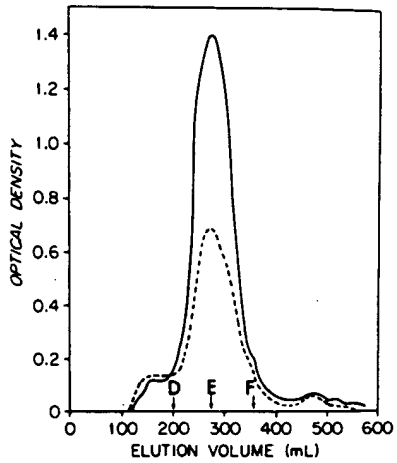

FIG. 5. *Final purification of toxin from CEX on a Sephadex G-200 column (2.5 by 97.5 cm) at pH 6.0. (D, E, and F) Points where samples were taken for electrophoresis. OD at 280 nm (——); OD at 260 nm (---).*

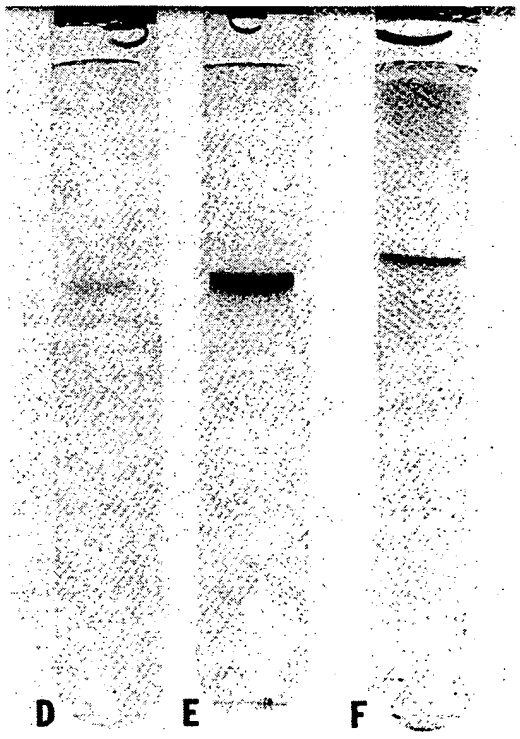

FIG. 6. *Polyacrylamide gel electrophoresis of eluate from Sephadex G-200 at elution volumes of 200 (D), 270 (E), and 350 ml (F).*

This fluid was next chromatographed on Whatman DE-52. A toxic fraction eluted frontally; increasing the ionic strength of the eluant buffer by adding NaCl resulting in the elution of a large nontoxic fraction with OD_{280}/OD_{260} less

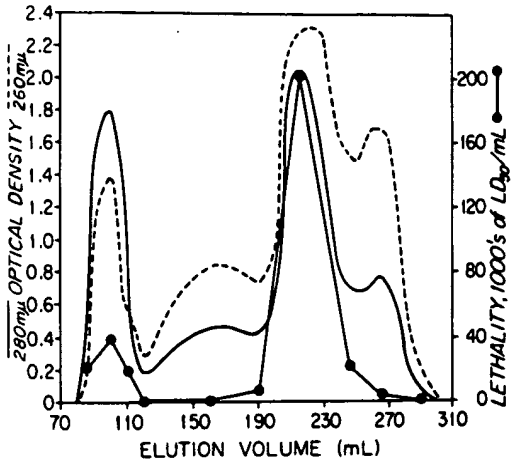

FIG. 7. *Gel filtration of CEX on a Sephadex G-50 column at pH 4.5.*

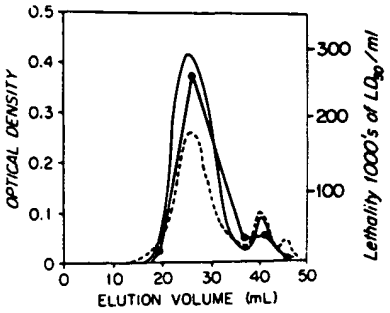

FIG. 8. *Final purification of toxin from CEX on a Sephadex G-200 column (1 by 30 cm) at pH 4.5. OD at 280 nm (——); OD at 260 nm (---); and lethality (●——●).*

than unity. The fractions from the toxic peak were pooled, precipitated with ammonium sulfate, dissolved in pH 4.5 acetate buffer, and chromatographed on Sephadex G-200. The toxin eluted in a single, nearly symmetrical peak at 1.8 × V_o, just as it had in the purification at pH 6.0. That is, after chromatography on DE-52 and precipitation with ammonium sulfate, the apparent MW of the toxic material, as measured by Sephadex gel filtration at pH 4.5, had changed from approximately 20,000 to approximately 80,000.

Gerwing et al. (8) have suggested that one possible reason for what they considered the aggregation of Sakaguchi toxin preparation is the use of repeated precipitations with ammonium sulfate. To evaluate this possibility, the purification at pH 4.5 was repeated, substituting concentration with Sephadex G-25 for the $(NH_4)_2SO_4$ precipitations. This led to a considerable reduction in concentration of toxin in all stages. The eluate from Sephadex G-50 again showed two toxic peaks. Both behaved similarly on DE-52, a toxic fraction eluting frontally but with an OD_{280}/OD_{260} ratio reduced to 1.4 to 1.6. The eluate, from DE-52, of the first peak from the G-50, was concentrated again with Sephadex G-25 and passed through a Sephadex G-200 column (1 by 30 cm) at pH 4.5 (Fig. 8). There were two toxic components in the eluate. The substances comprising both the major peak and a second smaller peak, with an OD_{280}/OD_{260} ratio less than 1.0, were toxic, that corresponding to the earlier larger peak of OD being the most lethal. The toxin of the earlier peak contained 20,000 LD_{50}/ml, whereas the latter one contained 8,000 LD_{50}/ml.

The second G-50 peak, after concentration with Sephadex G-25, was passed through Sephadex G-200 at pH 4.5 and gave similar results: two toxic peaks with the first containing the bulk of the activity.

Samples of the second peak eluted from Sephadex G-50 were run in an analytical ultracentrifuge. Results indicated that this fraction contained two major components. A rapidly sedimenting moiety was found to contain all of the toxicity present in the sample. The rate of sedimentation for this component was consistent with a MW of approximately 90,000. The slower component, with a MW of about 20,000, was nontoxic. The final product (the eluate from Sephadex G-200) contained only the rapidly sedimenting toxic moiety.

To further reduce the possibility of aggregation of the toxin molecules, the eluting buffer was changed to 0.025 N acetate; guanidine hydrochloride was added to this buffer in a concentration of 0.05 M. Elution patterns obtained were indistinguishable from those shown in Fig. 7 and 8 where guanidine was not used.

Samples were also purified using 0.05 N acetate buffer at pH 5.3. This gave a small reduction in the OD_{260} of the first peak extracted from Sephadex G-50, with a corresponding increase in the OD_{260} of the last peak. However, there was no shift of toxicity from the first to the second peak, and no precipitate formed in the fractions collected from the second G-50 peak. Toxic samples from the first peak were pooled and chromatographed on Whatman DE-52 at pH 5.3 and then precipitated and chromatographed on Sephadex G-200. The final product obtained at pH 5.3 eluted from Sephadex G-200 at 1.8 × V_o, just as it had at the higher and lower pH values.

Purification of released toxin from culture filtrate. Chromatography on Sephadex G-50 at

pH 4.5 (Fig. 9) gave two toxic components, the first at the V_o and the second considerably later. The first toxic peak contained 2.5 times the activity as measured in LD_{50}/ml, of the second peak, although the second peak gave higher OD readings.

Ion-exchange chromatography of each toxic peak on Whatman DE-52 at pH 4.5 resulted in the frontal elution of a toxic component with an OD_{280}/OD_{260} ratio of 1.9 or greater. These toxic fractions were precipitated by the addition of 70% saturated ammonium sulfate and the dissolved precipitates were chromatographed on Sephadex G-200. In each case, the result was elution of a single toxic peak at $1.8 \times V_o$. Similar purifications carried out at pH 5.3 and 6.0 resulted in nearly complete elimination of the lethality of the second eluate peak from Sephadex G-50.

The addition of 0.05 M guanidine hydrochloride to each of the eluting buffers used did not change the behavior of the toxin.

An attempt was made to carry out the purification without using precipitation with $(NH_4)_2SO_4$, but, due to the large volumes, the low concentrations of toxin, and the relative inefficiency of the Sephadex G-25 method of concentration, this attempt had to be abandoned. Instead, samples of culture filtrate were chromatographed at both pH 4.5 and 6.0 on Sephadex G-200 before treatment of any kind, other than removal of cells and cell debris (Fig. 10). At pH 6.0, the toxic activity was found in the peak at 290 ml ($1.8 \times V_o$). At pH 4.5, significant toxic activity was found at the V_o of the Sephadex G-200, at $1.8 \times V_o$ (290 ml), and at $2.7 \times V_o$ (435 ml). The last peak, at pH 6.0, was nontoxic.

Lethality. The toxin preparations contained from 6.9×10^4 LD_{50}/mg to 6.4×10^5 LD_{50}/mg N before trypsinization and 1.2×10^6 LD_{50}/mg to 8.0×10^7 LD_{50}/mg N after trypsinization.

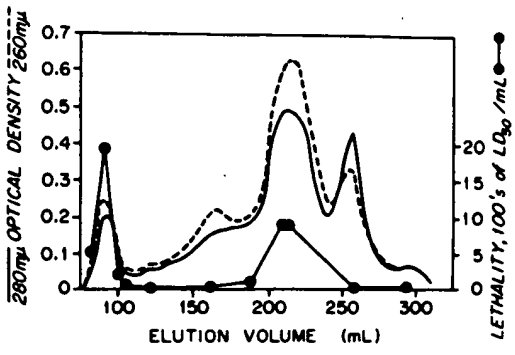

FIG. 9. *Gel filtrations of culture filtrate on a Sephadex G-50 column (2.5 by 40 cm) at pH 4.5.*

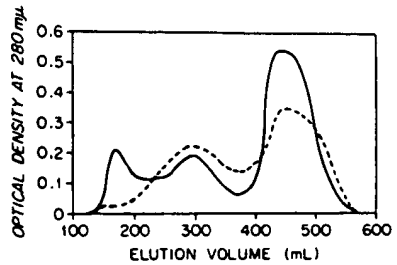

FIG. 10. *Gel filtration of culture filtrate on a Sephadex G-200 column (2.5 by 97.5 cm). pH 4.5 (——); pH 6.0 (---).*

MW determination of purified toxin. To eliminate variables introduced by variations in size and packing of columns, $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of a given protein and V_t is the total volume of the column, was plotted against the log of the known MW of the protein (Fig. 11). The method of least squares was used to calculate the equation of the straight line best fitting the values obtained for the calibrating proteins. Then, plotting the K_{av} for the toxin of *C. botulinum* type E on the same line at its value of 0.35, one finds on the abscissa the projected MW of 86,000 for the purified protein. The same result was obtained for both trypsinized and untrypsinized samples of toxin.

Gel filtration in 6 M guanidine hydrochloride. Treatment with 6 M guanidine hydrochloride resulted in a complete loss of toxic activity. The elution pattern of toxin in 6 M guanidine hydrochloride is the solid line of Fig. 12. There are at least four small peaks with K_{av} between 0.33 and 0.60, and one large peak with a K_{av} of 0.75.

Pretreatment of the toxin with 2-mercaptoethanol and iodoacetic acid eliminates the small peaks and gives a single peak with a K_{av} of 0.75 (dashed line of Fig. 12). The shoulder on the right side of the peak corresponds to the elution volume of the 2-mercaptoethanol and iodoacetic acid when they are run alone. These data suggest that the toxin with a MW of 86,000 can be split into components of lower MW under conditions which usually do not break peptide bonds.

Stability of the toxin. A sample of purified retained toxin was stored in pH 6.0 phosphate buffer for up to 1 year at 4 C. During the time it lost more than 60% of its original lethality. The results of gel filtration of this sample on Sephadex G-200 are shown in Fig. 13. There was a major peak with a K_{av} of 0.23 and a smaller peak with a K_{av} of 0.74, plus a shoulder on the first peak at the V_o, and apparently components

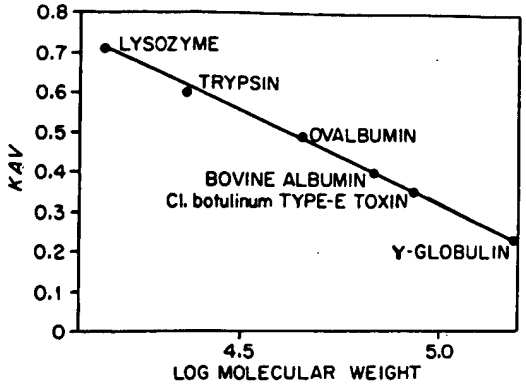

FIG. 11. *MW determination by gel filtration on Sephadex G-200 column. MW values for proteins other than C. botulinum toxin are literature values.*

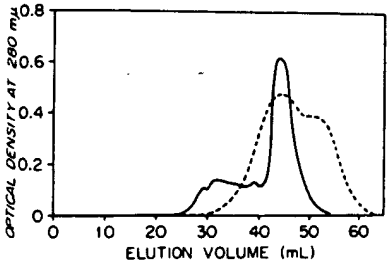

FIG. 12. *Gel filtration of purified toxin on a Sephadex G-200 column (1 by 30 cm) in 6 M guanidine hydrochloride. Purified toxin (——); purified toxin, reduced and alkylated (---).*

of several other sizes as well. Toxic activity was found to be present in the peaks corresponding to the K_{av} of 0.23 and 0.74 and the shoulder at the V_o. This suggests that the bulk of the toxin was present in a form with a MW of 160,000, with smaller amounts of toxin with MW of 12,000 and over 200,000.

DISCUSSION

At pH 6.0, a highly purified toxin could be prepared from the CEX of *C. botulinum* type E. The homogeneity of this preparation was demonstrated by the formation of a single symmetrical peak on Sephadex G-200, and by the presence of a single band on polyacrylamide gel electrophoresis at pH 4.0 and 8.0. The ratio of the OD at 280 nm to that at 260 nm was 2.0.

The same procedure carried out at pH 6.0 on the culture filtrate gave essentially similar results. The toxic eluate from Sephadex G-50 contained a yellow pigment which was separable by ion-exchange chromatography. Although the concentration of toxin was lower in the final preparations from culture fluid than in those from bacterial CEX, filtration through Sephadex G-200 indicated that the MW of both preparations were 86,000. This confirms the finding of Kitamura (10), that the origin of the toxin is not a significant factor in the size of the final product isolated. The toxin released into the culture medium appears to be the same as that extracted from intact cells.

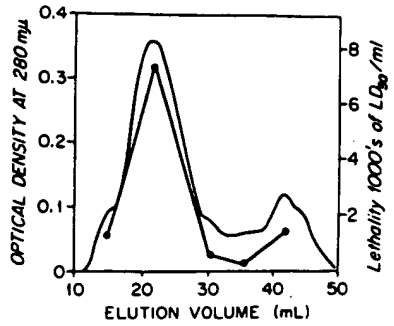

FIG. 13. *Gel filtration of aged purified toxin on a Sephadex G-200 column (1 by 30 cm) at pH 6.0. Toxin had been stored in solution at 4 C for 6 months. OD (——); lethality (●——●).*

When the purification of CEX was carried out at pH 4.5, the toxic activity was eluted from the Sephadex G-50 at two points. The major part of the toxic material was washed from the column quite late, which suggests that it had a LMW. Yet, further purification yielded a toxic molecule with a MW of 86,000. Ultracentrifugal analysis suggested that the toxic component in the eluate from Sephadex G-50 also had a MW close to this figure.

A possible explanation for this seeming disagreement is that at least a portion of the toxin in the crude preparation had a LMW, but that this form is not stable. This LMW toxin may be in equilibrium with a polymer or aggregate of a higher MW, the equilibrium being affected by other components of the mixture. If the gel filtration separated, or partially separated, the toxin from some substance that stabilized it in the form with a LMW, the result would be a shift to a form with a higher MW.

This hypothesis is supported by the behavior of the samples which were concentrated with Sephadex G-25 instead of $(NH_4)_2SO_4$. In these samples, the toxin with a LMW persisted throughout the purification procedure, albeit in low concentrations, so that a toxic fraction eluted from the final adsorption onto Sephadex G-200 in a volume suggesting a MW of around 12,000. This held true whether the fraction passed through the ion-exchanger and Sephadex G-200 came originally from the first or the second toxic peak eluted from Sephadex G-50. Both these peaks were ultimately found to con-

sist of over 90% HMW toxin and a small amount of LMW toxins. The HMW toxin peak had an OD_{280}/OD_{260} ratio of 2.0, while the LMW toxin peak had a ratio of 1.3.

These results support Gerwing's contention that the use of $(NH_4)_2SO_4$ precipitation tends to cause aggregation of the toxin. The LMW toxin prepared by us had notable similarities to the Gerwing preparation, for which her group calculated a MW of 14,000 to 16,000. Our extrapolated value of 12,000 seems to be in reasonable agreement. Another similarity between the two preparations is the OD_{280}/OD_{260} ratio, but our results do not support Gerwing's finding that such a preparation is free of RNA. On the contrary, our results suggest that the presence of impurities may very well permit the toxin to exist in a LMW form. The trypsinized toxin prepared by Emodi and Lechowich (3) was found on gel filtration to exist in two LMW forms, 5,000 and 9,000, but their preparation was not homogeneous on electrophoresis. This may be taken as further evidence that the toxin exists in LMW forms only when in the presence of stabilizing impurities, although the digestion by RNase and the high OD_{280}/OD_{260} ratios Emodi and Lechowich obtained make it unlikely that the stabilizer was RNA.

Gerwing (5, 6) reported that the use of low concentrations of guanidine salts reduced the tendency of her preparation of LMW to form aggregates. We were unable to confirm this finding. The addition of 0.05 M guanidine hydrochloride to our buffers did not reduce the high yields of HMW toxin nor increase the yield of LMW toxin. Stronger guanidine hydrochloride (6 M) caused our HMW toxin to deaggregate, but, with this concentration of guanidine, the toxic activity was destroyed. Gel filtration of HMW toxin in 6 M guanidine hydrochloride yielded at least five peaks. The largest peak eluted in a volume which suggested a MW of around 12,000. The identity of this peak with our LMW toxin was possible, but could by no means be considered proven since the eluate was nontoxic. In addition, guanidine, by rupturing hydrogen bonds, reduces the protein molecule to a random coil, so that the elution behavior could be altered by a change in the shape of the molecule.

The fact that not all the toxin was reduced to components of approximately 12,000 MW suggests that factors other than hydrogen bonding play a part in the aggregation of the toxin. When samples of toxin in 6 M guanidine were treated with 2-mercaptoethanol and iodoacetic acid, a procedure which reduces and alkylates sulfhydryl bonds, the peaks associated with substances of a higher MW disappeared, and a single elution peak was obtained at a volume indicating a MW of around 12,000.

A report has appeared recently showing the dissociation of a trypsinized 150,000-MW toxin into components of 50,000 and 102,000 MW under reducing conditions (2). These components were not alkylated after reduction, however, and the possibility therefore remains that some recombination may have taken place. Both hydrogen bonding and covalent bonding appear, therefore, to be factors in the aggregation of the toxin. This may explain why preparing LMW toxin was so difficult, at least under the conditions studied.

Since these findings indicated that the MW of the toxin isolated was affected by conditions of the purification scheme, untreated samples of culture filtrate were chromatographed directly on Sephadex G-200 columns. These studies revealed toxic activity associated with fractions of at least three MW, 12,000, 86,000, and 200,000 or more. This last figure corresponds to toxic activity eluting in the V_o volume, so that only a lower limit can be stated. The heaviest fraction may correspond to either the 12*S* toxin assigned a MW of 350,000 by Kitamura et al. (11) or to the 14*S* toxic moiety prepared by Schantz and Spero (16).

Further evidence for the aggregation of the toxin is its behavior with age. When preparations of purified 86,000-MW toxin were stored for long periods, both their lethality and homogeneity gradually decreased. Gel filtration of a sample of toxin which had been stored for 6 months showed that the bulk of the preparation was in a form with a MW of 150,000; a toxic component with a MW of 12,000 was present in comparatively small amounts. It is also possible that a toxic form with a MW over 200,000 was present, but that it was separated incompletely from the 150,000-MW form.

We were unable to confirm the finding by Kitamura et al. (12) of an E_δ component, a nontoxic fraction with a MW of 150,000, which separated from their 12*S* toxin at pH 8.0. Our 86,000-MW toxin was homogeneous on electrophoresis at pH 4.0 and pH 8.0, and could not be resolved into two components by ion-exchange chromatography at pH 8.0. It is possible that E_δ is less strongly bound to a 86,000-MW toxin than to the larger form found by Kitamura et al. and that, therefore, it separated from the toxin at an early stage of the purification process.

The possibility that all of the type E toxins characterized thus far represent different degrees of polymerization of the same molecule is attractive. The form assigned a MW of 9,000 by

Emodi and Lechowich thus may be identical to the form to which we have tentatively assigned a MW of 12,000. The fact that Gerwing (7) arrived at a figure of 14,000 to 18,000 for her LMW preparation by ultracentrifugal analysis rather than by gel filtration would make the agreement among the three sets of figures reasonably good.

Although the present study found no evidence for an even smaller monomer, the possibility remains that the toxin with a MW of 5,000 found by Emodi and Lechowich is the monomer and the 9,000 to 16,000 form is a dimer or trimer of this.

This idea of variable polymerization is made more reasonable by the present finding of a previously unrecorded MW of 86,000 for a toxic moiety. This figure is suggestively close to one-half the value of 150,000 assigned by Sakaguchi et al. (15) to their E_α component, and even closer to one-fourth the MW of 350,000 they gave for their 12S protoxin. This dissociation of the 86,000 moiety into components of 12,000, which has been demonstrated, would complete the range.

Until a method is devised for obtaining reasonably pure toxin of LMW, the possibility cannot be ruled out that the toxin activity of the LMW preparations is due to the presence of a HMW toxin as a contaminant. A lethality of 10^4 LD_{50}/mg N can be obtained from a preparation of pure LMW toxin, but this same figure can be obtained from a preparation containing 99% of a nontoxic LMW component and 1% HMW toxin with a lethality of 10^6 LD_{50}/mg N. Under these circumstances, the HMW toxin might be very difficult to detect.

Our results support the finding of Sakaguchi et al. (15) that the MW of the toxin is unchanged by the action of the proteolytic enzyme trypsin. We found identical elution patterns from Sephadex G-200 for trypsinized and untrypsinized samples of our 86,000-MW toxin.

ACKNOWLEDGMENT

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Purification of *Clostridium botulinum* Type F Progenitor Toxin

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Clostridium botulinum type F progenitor toxin was purified to a homogeneous state as judged by gel filtration on Sephadex G-200, ultracentrifugation, and disc electrophoresis. The sedimentation constant, corrected to water at 20 C, of type F progenitor toxin was determined to be 10.3 and the molecular weight to be 235,000 by ultracentrifugation at pH 6.0. The purified toxin contained a toxicity of 1.2×10^8 50% lethal doses/mg of N. In agar gel double diffusion, it formed two precipitin lines at pH 6.0. The progenitor toxin of type F differs from that of type A in that it contains no hemagglutinin and from that of type E in that it is not activable.

Møller and Scheibel (17) isolated a then new type of *Clostridium botulinum* from an outbreak of human botulism which occurred in Langeland, Denmark. Dolman and Murakami (8) designated the strain as *C. botulinum* type F and reported that the strain was relatively low in toxigenicity and that its toxin was relatively unstable.

Of the seven types from A through G now known for *C. botulinum* toxin, progenitor toxins, implying a natural state in which the toxin appears in real life as an identifiable entity (15), of types A (1, 10, 13) and E (12) have been purified and characterized. Those of types B (9, 14), C (4), and D (5) have been partially purified. A smaller molecular-sized derivative toxin (15) of type B has been purified and characterized (2, 7). There has been no report dealing with purification of type F progenitor or derivative toxin.

The present investigation was undertaken to establish procedures for purifying type F progenitor toxin and to compare it with those of other types in molecular structure and other respects.

MATERIALS AND METHODS

Strain. *C. botulinum* type F, strain Langeland, was used. A suspension of approximately 1,000 viable spores/ml in 0.05 M acetate buffer, pH 5.0, was kept in a frozen state and inoculated directly into the medium for toxin production.

Toxin production. The medium for toxin production consisted of 1.0% glucose, 1.0% yeast extract (Oriental Yeast Kogyo Co., Osaka), 2.0% peptone (for toxin production; Mikuni Kagaku Sangyo Co., Tokyo), and 0.025% sodium thioglycolate, with pH 7.0 adjusted with a 10% NaOH solution before autoclaving. About 1,000 viable spores were inoculated into 5 liters of the medium in a flat-bottomed spherical flask. The culture was incubated at 30 C for 4 days.

Chemicals and reagents. SP-Sephadex, C-50, and Sephadex G-200, medium, were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Protamine sulfate (salmon sperm) and the reagents for polyacrylamide gel electrophoresis, i.e., acrylamide monomer, *N,N'*-methylenebisacrylamide, and ammonium persulfate, were obtained from Seikagaku Kogyo Co., Tokyo. The ultrafiltration apparatus and UM 10 membrane were purchased from Amicon Co., Lexington, Mass.

Determination of protein contents. Protein contents were determined by the method of Lowry et al. (16).

Determination of toxin potency. The time-to-death method, by intravenous injection of mice (3, 20), was applied. The linear correlation between the log dose of type F toxin and the log period in minutes from injection to death is shown in Fig. 1. Usually each sample was injected into three mice at 0.1-ml doses. When necessary, each of serial twofold dilutions of a material in 0.05 M acetate buffer, pH 6.0, was injected intraperitoneally into no less than four mice at 0.5-ml doses to calculate the 50% lethal dose (LD_{50}) in 4 days by using the method of Reed and Muench (18).

Agar gel double diffusion. The method reported by Kitamura et al. (12) was used.

Antitoxin type F. The purified progenitor toxin was treated with 0.4% formalin at pH 6.0 and 30 C for 7 days. A 0.1-mg portion of the toxoid emulsified in an equal volume of aluminum phosphate gel was injected subcutaneously into each rabbit. The toxoid-adjuvant mixture was injected three times at 3-day intervals. Four weeks after the third injection, 0.1-mg doses of purified toxin were injected, also subcutaneously, twice at a weekly interval. The animals were bled 10 days after the final injection. The serum was fractionated with ammonium sulfate; the resulting immunoglobulin G fraction was further gel-filtered on a Sephadex G-200 column.

Polyacrylamide gel electrophoresis. Using the method reported by Reisfeld et al. (19), 4.5% gel at pH 4.0 was prepared. The gel columns were stained in an amido black 10B solution for about 18 h and then destained with 7% acetic acid. Neutral red was used as a marker dye to measure the relative mobility.

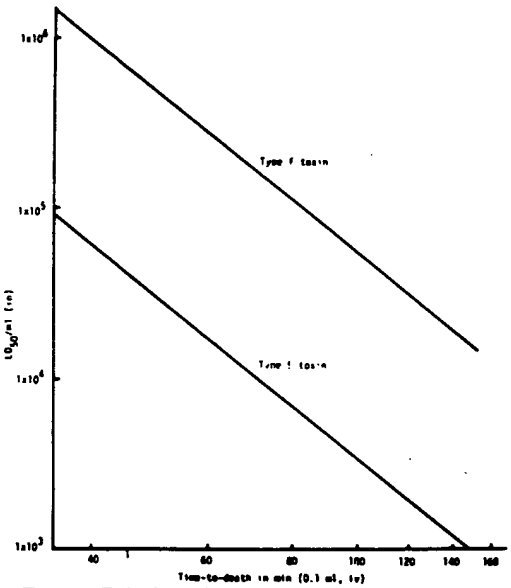

FIG. 1. *Relationship between the dose in intraperitoneal (ip) LD_{50} and the time from intravenous (iv) injection to death. Each dilution of type F toxin in 0.05 M acetate buffer, pH 6.0, was injected intravenously into 5 mice at a 0.1-ml dose.*

Ultracentrifugal analysis. A Beckman model E ultracentrifuge was used. The homogeneity of the purified toxin was proved by the band-sedimentation method with ultraviolet absorption optics. A sample (19 μg/5 μliters) in 0.1 M acetate buffer, pH 6.0, containing 1.0 M NaCl was centrifuged at 56,100 rpm at 20.0 C. The sedimentation equilibrium method (21) was employed to determine the molecular weight of the purified toxin. The toxin was centrifuged in 0.1 M acetate buffer, pH 6.0, containing 0.1 M NaCl at 5,227 rpm at 16.3 C in a double-sector cell.

RESULTS

Purification of type F progenitor toxin. (i) Step 1. The whole culture at pH 5.6 was acidified to pH 4.0 with 3 N H_2SO_4. The mixture was allowed to stand overnight at room temperature. The supernatant fluid was siphoned off, and the bottom fluid was centrifuged at 3,800 $\times$ *g* for 10 min. The packed precipitate was resuspended in 0.2 M phosphate buffer, pH 6.0.

(ii) Step 2. One-fourth volume of a saturated ammonium sulfate solution was added slowly to the suspension while the mixture was kept stirring on a magnetic stirrer. The mixture was allowed to stand at room temperature for 30 min and then was centrifuged. The supernatant fluid was added with solid ammonium sulfate (472 g/liter) to make 70% saturation. The precipitate formed was collected by centrifugation at 3,800 $\times$ *g* for 10 min and dissolved in 0.2 M phosphate buffer, pH 6.0. Ammonium sulfate fractionation was repeated again; the second precipitation was performed at 65% saturation (430 g/liter). The precipitate containing the most toxic activity was resuspended in 0.05 M acetate buffer, pH 4.5, and dialyzed against 20 volumes of the same buffer in the cold.

(iii) Step 3. The precipitate formed during dialysis was collected by centrifugation at 8,600 $\times$ *g* for 10 min and extracted with 0.25 M NaCl-0.1 M acetate buffer, pH 4.5. The extract recovered little toxicity and was therefore discarded. The residue was extracted again with 0.5 M NaCl-0.1 M acetate buffer, pH 4.5. The toxic extract was added dropwise with a freshly prepared 2% protamine solution. The amount of protamine to be added was estimated from the absorption of the extract at 260 nm (A_{260}); addition with 2.0 ml of the 2% protamine solution to 100 ml of extract with an A_{260} of 1.0, containing about 3 mg of protein/ml, gave a satisfactory result. The precipitate formed was removed by centrifugation at 6,800 $\times g$ for 10 min. The supernatant was adjusted to pH 4.2 with 0.5 M NaCl-0.1 N acetic acid and allowed to pass through a column (1.5 by 10 cm) of SP-Sephadex, C-50, equilibrated with 0.5 M NaCl-0.1 M acetate buffer, pH 4.2.

(iv) Step 4. The percolate from the SP-Sephadex column was diluted with 0.1 M acetate buffer, pH 4.2, to make the NaCl concentration to 0.1 M. It was applied to a column of SP-Sephadex, C-50 (1.5 by 10 cm), and equilibrated with 0.1 M NaCl-0.1 M acetate buffer, pH 4.2. The toxin adsorbed onto the column was eluted at an NaCl concentration of approximately 0.35 M (Fig. 2). The toxic fractions were pooled and concentrated by ultrafiltration through a UM 10 membrane.

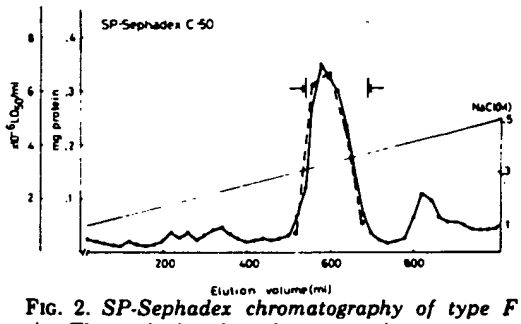

FIG. 2. *SP-Sephadex chromatography of type F toxin. The toxin fraction after protamine treatment was applied to an SP-Sephadex, C-50, column and eluted by linear gradient in NaCl from 0.10 to 0.5 M in 500 ml of 0.10 M acetate buffer, pH 4.5. Ten-milliliter fractions were collected. Symbols: protein content, ●; toxic activity, O; NaCl concentration, ——. Fractions indicated by arrows were pooled.*

(v) Step 5. The concentrated toxic fraction was applied to a column of Sephadex G-200 (2.5 by 190 cm). A major protein peak was eluted after a minor one (Fig. 3). The front peak was resolved into two bands in disc electrophoresis; the retarded peak formed a single band. The toxic fractions of the major peak showing a single band in disc electrophoresis were pooled and concentrated by ultrafiltration through a UM 10 membrane.

All overall purification of about 50-fold was accomplished from the acid precipitate of the whole culture (Table 1).

Examinations of the purified type F progenitor toxin for homogeneity and some other properties. (i) Gel filtration. The purified material was eluted from a column of Sephadex G-200 with 0.1 M acetate buffer-0.3 M NaCl, pH 4.2, as eluant in a single symmetrial peak. The toxicities per milligram of nitrogen of representative fractions were on the same level throughout the peak (Fig. 4). The elution volume of the purified toxin from the column coincided to that of the toxin in the culture supernatant.

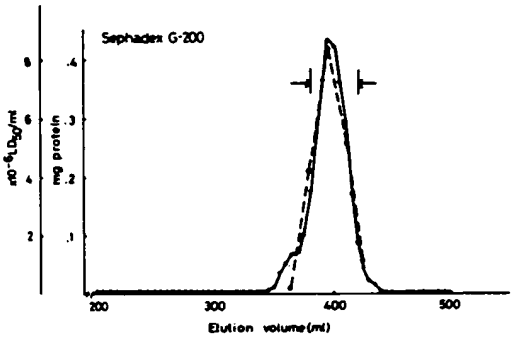

FIG. 3. *Gel filtration of type F toxin on Sephadex G-200. The toxic fraction eluted from SP-Sephadex and concentrated to 10 ml was applied to a Sephadex G-200 column (2.5 by 189 cm) equilibrated with 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl and eluted with the same buffer. Five-milliliter fractions were collected at a flow rate of 18 ml/h. Symbols: protein content, ●; toxic activity, ○. Fractions indicated by arrows were pooled.*

(ii) Disc electrophoresis. The purified type F toxin showed a single band with a relative mobility of 0.48 to the marker dye.

(iii) Agar gel diffusion. Two distinct precipitin lines were formed with any of the serial dilutions of the purified toxin and the antipurified type F toxin rabbit immunoglobulin G (Fig. 6).

(iv) Toxin potency. The toxicity of the purified material determined by the intraperitoneal injection method was 1.2×10^8 LD_{50}/mg of N. No increase in the toxicity resulted from the tryptic treatment of the purified toxin at pH 6.0.

(v) Direct hemagglutinin. No hemagglutinin activity was detected with the purified type F toxin at a concentration of 0.12 mg/ml against chicken erythrocytes at either 20 or 6 C; the crude culture supernatant showed slight activity (Table 2).

(vi) Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the purified toxin is shown in Fig. 7. The maximal absorption was at 278 nm with small shoulders at 253, 259, and 283 nm; the minimum was at 250 nm. The A_{278}:A_{250} ratio was 2.86. The A_{278} (1%) was 12.0 from the protein content in the bovine serum albumin equivalent.

(vii) Ultracentrifugation. The sedimentation velocity method demonstrated the homogeneity of the purified toxin with a sedimentation coefficient corrected to water at 20 C ($s_{20,w}$) of 10.3. From an assumed partial specific volume of 0.749 ml/g, the molecular weight of type F progenitor toxin was calculated to be 235,000.

DISCUSSION

Purification of *C. botulinum* type F progenitor toxin was accomplished for the first time. Since

TABLE 1. *Purification of C. botulinum type F progenitor toxin*

Step	Vol (ml)	Total		× 10^8 LD_{50}/mg of N[a]	Recovery (%)
		Protein (mg)	Toxicity (× 10^7 LD_{50})		
Whole culture	10,000	ND[b]	222	ND	100
Acid precipitate	700	4,641	200	2.7	91
Ammonium sulfate precipitate	50	521	189	2.7	86
Extract in 0.5 M NaCl	60	183	150	51.2	68
Protamine supernatant	75	63	85	84.2	43
SP-effluent	15	30	57	118	26
G-200 effluent	6	13.5	29	133	13

[a] LD_{50} per milligram of protein × 6.25.
[b] ND, Not determined.

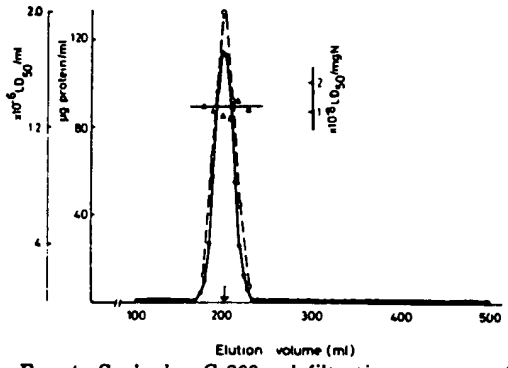

FIG. 4. *Sephadex G-200 gel filtration patterns of purified type F toxin. A sample of 3.3 mg in protein was applied to a column (2.5 by 98 cm) of Sephadex G-200 and eluted with 0.3 M NaCl-0.1 M acetate buffer, pH 4.2. Fractions (5.2 ml) were collected at a flow rate of 15 ml/h. Symbols: protein content, ●; toxic activity, O; specific activity, ▲. The arrow indicates the elution position of the toxin in the culture supernatant from the same column.*

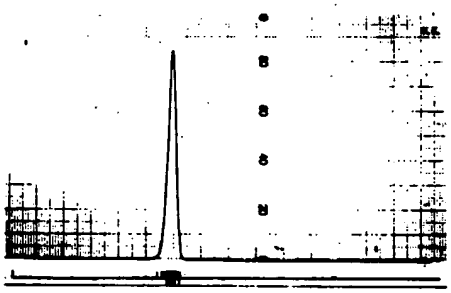

FIG. 5. *Disc electrophoretic pattern of purified type F toxin. A sample of 80 μg in protein was electrophoresed at pH 4.0 for 210 min. The current applied was 2.5 mA per tube. The stained protein bands were monitored by a densitometer (type FDA IV, Fujiriken Co., Tokyo) at 600 nm.*

types A and E progenitor toxin are known to be composed of two components, toxic and atoxic, and to dissociate into the two components under slightly alkaline conditions (7, 13), in the present investigation, aiming at isolating the intact type F progenitor toxin, care was taken to avoid possible molecular dissociation during purification. All the procedures were performed under acidic conditions to isolate the same natural toxin as that appearing in culture. As the purified toxin was demonstrated by gel filtration to have a molecular size identical to that of the toxin in the culture supernatant, none of the procedures employed altered the molecular size during purification.

The purified toxin behaved as a homogeneous protein in all the procedures for testing the homogeneity, but agar gel diffusion with serial dilutions of the preparation gave two precipitin lines. The fact that the two lines were formed at any dilution of the toxin may suggest that type F progenitor toxin is also composed of two components, probably toxic and atoxic components, like types A and E progenitor toxin (15).

Trypsinization of type F toxin at pH 6 did not induce any increased toxicity. This must have been due to spontaneous activation during the 4 days of incubation with the protease(s) produced by the organisms themselves. The toxins of proteolytic *C. botulinum* are not activable, except a type B strain Okra which produces both activable toxin (11) and an activating enzyme (6). This may not necessarily reflect different proteases produced by these organisms, but may suggest different molecular structures of these toxins.

The purified type F progenitor toxin had an $s_{20,w}$ of 10.3 and a molecular weight of 235,000, which is significantly smaller than those of types A (900,000 [15]) and E (350,000 [12]) toxin. The specific toxicity of type F progenitor toxin was 1.2×10^8 LD_{50}/mg of N, which is higher than that of type E (8×10^7 LD_{50}/mg of N [12]), but lower than that of type A (2.4×10^8

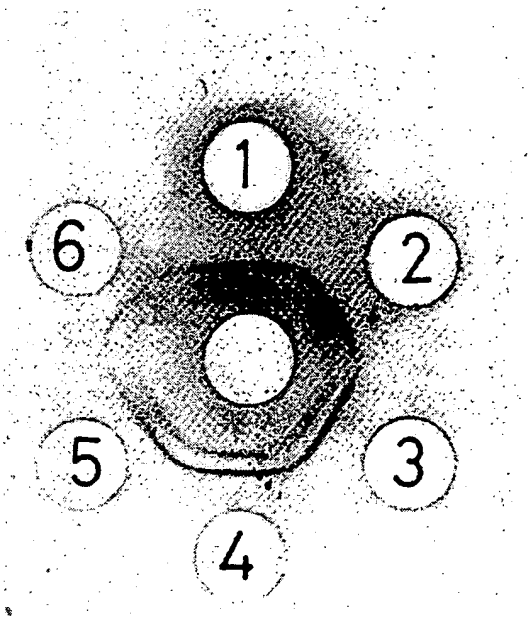

FIG. 6. *Agar gel double diffusion tests with purified type F toxin. Center well: anti-type F toxin globulin; lateral wells: purified type F toxin; 1: 625 μg/ml; 2: 312 μg/ml; 3: 156 μg/ml; 4: 78 μg/ml; 5: 39 μg/ml; 6: 19.5 μg/ml. The gel was washed with phosphate-buffered saline, pH 7.3, and the precipitin lines were staind with thiazine red.*

TABLE 2. *Hemagglutinin activities of types A and F progenitor toxin*[a]

Toxin type	Temp (C)	Concn (μg of protein/ml)										
		120	60	30	15	7.5	3.8	1.9	0.94	0.47	0.23	0.12
A[b]	6			+	+	+	+	+	+	+	+	-
	20			+	+	+	+	+	+	+	-	-
F[c]	6	-	-	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-	-	-	-
Supernatant[d]	6	+ (2×)[e]	+ (4×)	+ (8×)	+ (16×)	+ (32×)	+ (64×)	- (128×)	- (256×)	- (512×)	- (1,024×)	- (2,048×)

[a] The reaction mixture contained 0.5 ml of a sample diluted in 0.075 M NaCl-0.075 M phosphate buffer, pH 7.3, containing 0.25% bovine serum albumin and 0.05 ml of a 2.5% suspension of chicken erythrocytes.
[b] Crystalline type A toxin.
[c] Purified type F progenitor toxin.
[d] Supernatant of a 98-h culture of *C. botulinum* type F.
[e] Numbers in parentheses give dilution.

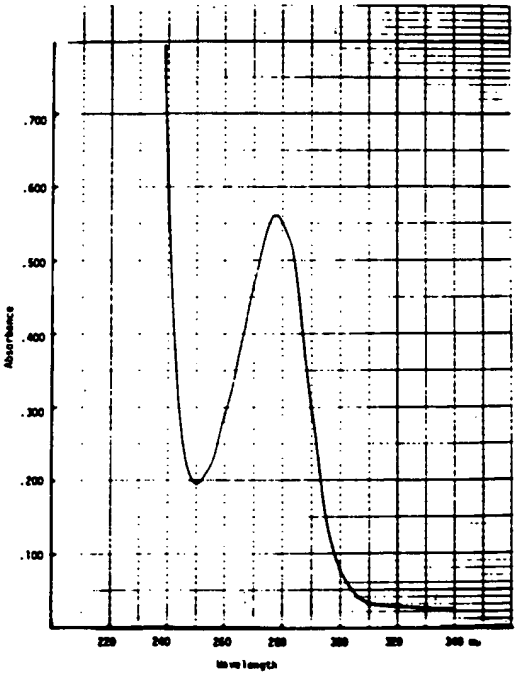

FIG. 7. *Ultraviolet absorption spectrum of purified type F toxin. A solution of 466 μg/ml in 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl was examined in a Hitachi spectrophotometer, type 124.*

LD_{50}/gm of N [15]) progenitor toxins. Type F progenitor toxin, like type E, contains no hemagglutinin, although the culture supernatant contains some activity.

To perform ion exchange chromatography of crude toxin at an acidic reaction, it was necessary to remove such strongly acidic substances as nucleic acids, since the presence of such substances caused precipitation of the toxin when equilibrated to pH 4.5 or below at a low salt concentration. Attempts were made, therefore, to remove such substances from the toxin material by treating with streptomycin, ribonuclease, diethylaminoethyl-Sephadex, or protamine. The treatment with protamine appeared to be most practicable for the purpose, as its effect was not significantly influenced by either the salt concentration or the pH value. The quantity of protamine to be added, however, should have been determined preliminarily in a small scale, because the acidic substance contents varied from one preparation to another and the quality of protamine also varied from lot to lot.

In the final Sephadex G-200 gel filtration, the column length was crucial for separation of the major toxin peak from the minor contaminant peak. It seems possible to separate these materials more efficiently by the recycling method of gel filtration.

ACKNOWLEDGMENTS

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Oral Toxicities of *Clostridium botulinum* Type C and D Toxins of Different Molecular Sizes

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Clostridium botulinum type C progenitor toxins of different molecular sizes, C-L (16S) and C-M (12S), were purified from cultures of strains 573, Stockholm, and CB-19. C-L toxin showed some hemagglutinin activity, whereas C-M toxin did not. Neither C-L nor C-M toxin was activated upon trypsinization. Molecular dissociation of purified type C-L and C-M toxins into toxic and nontoxic components was demonstrated by sucrose density gradient ultracentrifugation and diethylaminoethyl-Sephadex chromatography at pH 8.0. The molecular construction of type C progenitor toxin appears to be analogous to that reported for botulinum toxins of other types. C-L and D-L toxins showed higher oral toxicities to mice than did C-M or D-M toxin. Such higher oral toxicities were ascribed to the higher stabilities of these toxins in gastric and intestinal juices.

Progenitor toxins of *Clostridium botulinum* types A, B, E, and F, being the complex of toxic and nontoxic components, are orally more potent to mice than are the corresponding derivative toxins, dissociated toxic components, of 5 to 7S (17, 21). Type A-L and B-L toxins of 16S possessed particularly high oral toxicities as compared with type A-M, B-M, or F toxin of 10 to 12S (17). Such higher oral toxicities shown by larger molecular-sized progenitor toxins were ascribed to their higher resistance to gastric and intestinal juices. The oral toxicity of botulinum toxin hence depends on the complexity of the molecular construction and the resulting stability in the digestive tract of the host animal (26).

Susceptibilities of different animal species to oral administration of *C. botulinum* type C and D toxins have been reported (9, 18, 24, 27-29). No attention, however, has been paid to different molecular-sized toxins in the past feeding experiments. The present investigation demonstrates that strains of *C. botulinum* type C commonly produce 12S and 16S toxins as do types A (25), B (11), and D (13), and that the oral toxicities and stabilities of type C and D toxins differ depending upon the molecular size.

MATERIALS AND METHODS

Bacterial strains and toxin production. *C. botulinum* type C strains 573, Stockholm, and CB19, and type D strains 1873, 4947, and CB16 were used. Type C strain Stockholm was supplied by J. Müller, State Veterinary Serum Laboratory, Copenhagen. Type C strain CB19 and type D strain CB16, isolated from mink feed incriminated in an outbreak occurring in Hokkaido in 1975 and from soil in Tokyo at the time of an outbreak among wild ducks in 1973, respectively, were provided by S. Sakai, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo. Each strain was inoculated into a medium composed of 0.5% glucose-0.5% yeast extract-2.0% peptone-0.1% cysteine-hydrochloride (pH 7.2) to which was added 0.04 volumes of a salt solution of 0.02% magnesium sulfate-0.026% calcium chloride-0.1% potassium diphosphate-0.1% potassium monophosphate-1.0% sodium bicarbonate-0.2% sodium chloride. For toxin production, an overnight culture in cooked meat medium fortified with 1% ammonium sulfate, 1% glucose, 1% yeast extract, and 0.1% cysteine-hydrochloride (pH 7.4) was inoculated into the above medium (10 ml/5,000 ml), which was incubated for 3 days at 37°C.

Preparation of culture supernatant and cell extract for ultracentrifugal analyses. Portions (0.5 ml) of *C. botulinum* type C and D strains grown overnight in the fortified cooked meat medium at 37°C were each inoculated into 10 ml of saline in dialysis casing suspended in 215 ml of the same medium in a flask. After incubation for 3 days at 37°C, the supernatant of the culture in the dialysis casing was obtained by centrifugation for 20 min at 7,800 × *g*. The cell extract was prepared by resuspending the precipitated cells in 2.0 ml of 0.2 M phosphate buffer (pH 6.0) and centrifuging for 20 min at 7,800 × *g*.

Purification of progenitor toxins of *C. botulinum* types C and D. *C. botulinum* type C and D toxins were purified by essentially the same procedures as those employed for purification of type F progenitor toxin (14, 16) except the step of acid precipitation, in which the toxin was precipitated by adjusting the culture fluid to pH 4.0 with 3 N H_2SO_4 in the presence of added ribonucleic acid (0.4 mg/ml) as a precipitation aid (8).

Determinations of intraperitoneal and oral toxicities. The intraperitoneal mean lethal dose (i.p. LD_{50}) was determined by the time-to-death method by injecting mice intravenously with 0.1-ml doses (1, 22). Activation was studied by treating the toxin sample with trypsin at an enzyme-to-substrate ratio of 1:2 in 0.1 M acetate buffer (pH 6.0) for 30 min at 35°C.

The oral LD_{50} was calculated by the method of Reed and Muench (19) and expressed in the equivalent number of i.p. LD_{50}. Oral administration of toxin to mice was performed as described previously (17).

Tests for stability of the toxin in gastric and intestinal juices. Gastric and intestinal juices were obtained from 280- to 350-g Wistar strain male rats by inserting polyethylene tubing into the stomach and the duodenum, respectively (26). For the stability test, 1 volume of toxin (50 μg/ml) was incubated at 35°C in 4 volumes of gastric (pH 1.4) or intestinal juice (pH 7.5). Since type D progenitor toxin is activable, the toxin to be used for the stability test was treated with trypsin at a toxin-to-trypsin ratio of 2:1 in 0.1 M acetate buffer (pH 6.0) for 30 min at 35°C, and the trypsin was removed on soybean trypsin inhibitor-coupled Sepharose 6B prepared by the method of Feinstein (4).

Other procedures. Protein was determined by the method of Lowry et al. (12). Ultracentrifugation in a 5 to 20% linear sucrose density gradient was performed by the procedure reported elsewhere (25). Disc electrophoresis at pH 4.0 in 4.5% polyacrylamide gel was carried out by the method of Reisfeld et al. (20). Neutral red was used as a marker dye to measure the relative mobility.

RESULTS

Purification and some properties of type C and D progenitor toxins. At the final step of purification of the toxins of all strains of *C. botulinum* types C and D employed, two toxin peaks were eluted from a column of Sephadex G-200 at the positions corresponding to those of B-L (16S) and B-M (12S) toxins. The first eluted peak was named L toxin, and the second one was named M toxin. In disc electrophoresis at pH 4.0, M toxin of any strain gave a single band with a relative mobility of 0.48, while L toxin gave three to four bands. The parenteral toxicities of D-L and D-M toxins increased two to six times upon trypsinization, whereas those of type C strains remained almost at the same level. All L toxins purified from type C and D cultures showed some hemagglutinin activity against the chicken erythrocytes. The toxicities and hemagglutinin activities of purified type C and D toxins are summarized in Table 1.

Molecular dissociation of type C and D progenitor toxins into toxic and nontoxic components. Purified toxins were each centrifuged in a 5 to 20% sucrose density gradient in 0.05 M phosphate buffer (pH 6.0), at 123,000 × *g* for 7 h at 6°C. All C-L and D-L toxins sedimented to the same relative position as that of B-L toxin (16S), and all M toxins sedimented to that of B-M toxin (11.6S). The presence of both L and M toxins was demonstrated in the culture supernatant and cell extract of all strains except type D strain 1873, of which the toxin peak was broad and mainly at the same position as that of B-M toxin (Fig. 1). In sucrose density gradient ultracentrifugation at pH 7.0 or above, C-L toxin was resolved into toxic and nontoxic components

TABLE 1. *Toxicities and hemagglutinin activities of purified type C and D progenitor toxins*

Toxin type	Strain	Toxicity (i.p. LD_{50}/mg of protein)		Ratio[a]	Hemagglutinin activity[b]
		Before trypsinization	After trypsinization		
C-L	573	3.2×10^6	3.8×10^6	1.2	2^8
	Stockholm	4.6×10^6	4.3×10^6	0.93	2^8
	CB19	1.0×10^7	9.8×10^6	0.98	2^3
C-M	573	1.0×10^7	1.2×10^7	1.2	–[c]
	Stockholm	1.6×10^7	1.4×10^7	0.88	–
	CB19	1.9×10^7	1.8×10^7	0.95	–
D-L	4947	4.5×10^7	2.9×10^8	6.4	2^8
	1873	5.8×10^7	2.6×10^8	4.5	2^8
	CB16	3.0×10^7	1.2×10^8	4.0	2^8
D-M	4947	1.5×10^8	3.2×10^8	2.1	–
	1873	1.8×10^8	5.3×10^8	2.9	–
	CB16	3.9×10^8	1.4×10^9	3.5	–

[a] Ratio of toxicity before and after trypsinization.

[b] Reciprocal of the highest dilution of toxin at 0.1 mg/ml with 0.5% chicken erythrocyte suspension at room temperature.

[c] –, Negative at 1 mg/ml.

FIG. 1. *(A) Ultracentrifugal analyses of the cell extract of C. botulinum type C strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the cell extract of type C strain 573 contained 9.4×10^4 LD_{50}, that of strain Stockholm contained 3.2×10^5 LD_{50}, and that of strain CB19 contained 3.7×10^5 LD_{50}. (a) Type C strain 573; (b) type C strain Stockholm; (c) type C strain CB19. (B) Ultracentrifugal analyses of the culture supernatant of C. botulinum type C strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of strain 573 contained 0.8×10^4 LD_{50}, that of strain Stockholm contained 1.2×10^4 LD_{50}, and that of strain CB19 contained 8.0×10^5 LD_{50}. (d) Type C strain 573; (e) type C strain Stockholm; (f) type C strain CB19. (C) Ultracentrifugal analyses of the cell extract of C. botulinum type D strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the cell extract of strain 4947 contained 8.8×10^4 LD_{50}, that of strain 1873 contained 4.6×10^6 LD_{50}, and that of strain CB16 contained 4.2×10^6 LD_{50}. (a) Strain 4947; (b) strain 1873; (c) strain CB16. (D) Ultracentrifugal analyses of the culture supernatant of type D strains in sucrose density gradient (5 to 20%) in 0.05 M acetate buffer, pH 6.0. A 0.2-ml sample of the culture supernatant of strain 4947 contained 8.8×10^4 LD_{50}, that of strain 1873 contained 2.0×10^6 LD_{50}, and that of strain CB16 contained 6.0×10^5 LD_{50}. (d) Strain 4947; (e) strain 1873; (f) strain CB16.*

as reported with other types (11, 13, 25); the former sedimented to the same position as that of a toxic component (7S), and the latter sedimented to that of a nontoxic component (14S) of B-L toxin. The dissociation pattern of C-L toxin is shown in Fig. 2.

C-M toxin preparations purified from *C. botulinum* type C strains 573, Stockholm, and CB19 sedimented to the same relative position as that of the toxic component of B-L toxin in ultracentrifugation at pH 8.0, but no separation into toxic and nontoxic components was ob-

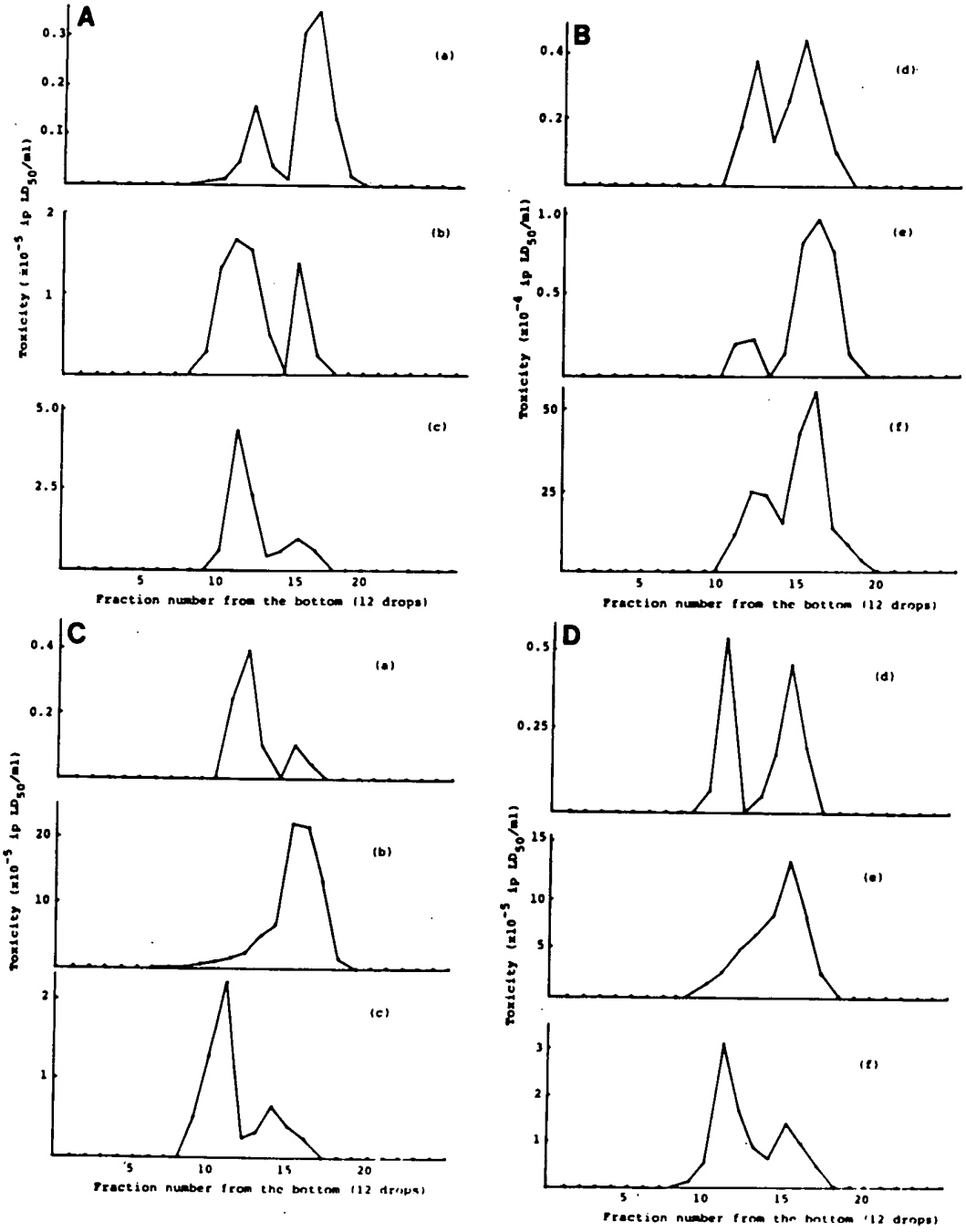

FIG. 1.

served. When purified C-M toxin was chromatographed on a column of diethylaminoethyl (DEAE)-Sephadex A-50 at pH 8.0, however, two protein peaks of approximately the same areas were separated (Fig. 3); the early eluted one was toxic, and the late eluted one was nontoxic, indicating that C-M toxin is constructed with two components, toxic and nontoxic, and dissociates into the two at pH 8.0.

Oral toxicities of botulinum type C and D toxins. The oral toxicities of type C and D toxins of different molecular sizes are shown in Table 2. The oral toxicities of C-L and D-L toxins (16S) of all strains were higher than those of C-M or D-M toxin; those of C-L toxins were

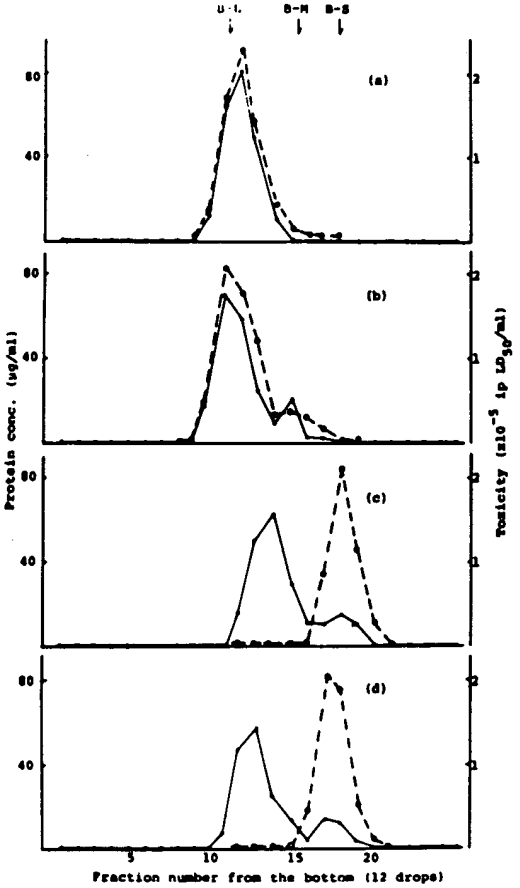

FIG. 2. *Ultracentrifugal analyses of the purified progenitor toxin of type C strain 573 in sucrose density gradient (5 to 20%) prepared in 0.05 M phosphate buffer, pH (a) 6.5, (b) 7.0, (c) 7.5, and (d) 8.0. A 0.2-ml sample of C-L toxin contained 85 μg of protein and 3.5 × 10⁵ LD50. B-L, B-M, and B-S indicate the sedimentation positions of B-L (16S), B-M (12S), and B-S (7S) toxins, respectively. Symbols: ●, Protein content; ○, toxicity.*

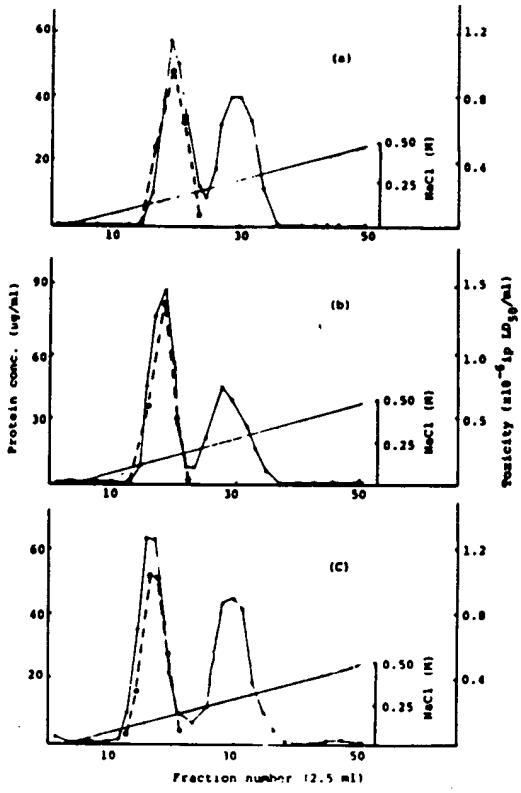

FIG. 3. *Chromatography of purified C-M toxin of type C strains 573, Stockholm, and CB19 on a DEAE-Sephadex A-50 column (1.5 by 10 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0. A 2.0-mg portion of toxin was applied and eluted with a linear gradient increase in NaCl concentration from 0 to 0.5 M in 100 ml of the buffer. (a) Strain 573; (b) strain Stockholm; (c) strain CB19. Symbols: ●, Protein content; ○, toxicity; ——, NaCl concentration.*

TABLE 2. *Oral toxicities of C. botulinum type C and D progenitor toxins*

Toxin type	Strain	Oral toxicity (i.p. LD_{50})	Ratio[a]
C-L	573	6.7×10^3	2.5
	Stockholm	6.5×10^3	2.4
	CB19	2.7×10^3	1.0
C-M	573	2.7×10^5	100
	Stockholm	0.95×10^5	35
	CB19	1.2×10^5	44
D-L	4947	3.5×10^4	13
	1873	7.6×10^4	28
	CB16	7.6×10^4	28
D-M	4947	0.96×10^5	35
	1873	1.3×10^5	48
	CB16	8.8×10^5	326

[a] Oral toxicity of type C-M toxin of strain 573 was taken as 100.

15 to 45 times higher than those of C-M toxins. No significant difference was found between C-M and D-M toxins in the oral toxicity expressed in equivalent mouse i.p. LD_{50}.

Stabilities of type C and D toxins in gastric and intestinal juices. Stabilities of type C and D toxins of different molecular sizes in gastric and intestinal juices were tested (Tables 3 and 4). C-L and D-L toxins of all the strains were more resistant to either gastric or intestinal juices than were C-M and D-M toxins. D-M toxin was more resistant to treatment with intestinal juices than was C-M toxin. Less than 0.02% of the original C-M toxicity remained after a 180-min exposure to intestinal juices at 35°C.

TABLE 3. *Stabilities of type C and D progenitor toxins in rat gastric juice*

Toxin type	Strain	Remaining toxicity (%) after[a]	
		40 min	120 min
C-L	573	79	16
	Stockholm	76	68
	CB19	82	68
C-M	573	3.7	0.8
	Stockholm	12	1.6
	CB19	10	1.1
D-L	4947	100	66
	1873	100	27
	CB16	79	45
D-M	4947	5.8	1.2
	1873	2.0	0.2
	CB16	2.6	0.6

[a] Incubation at 35°C.

TABLE 4. *Stabilities of type C and D progenitor toxins in rat intestinal juice*

Toxin type	Strain	Remaining toxicity (%) after[a]	
		60 min	180 min
C-L	573	109	78
	Stockholm	100	79
	CB19	85	80
C-M	573	0.38	<0.02
	Stockholm	0.85	<0.02
	CB19	0.95	<0.02
D-L	4947	81	61
	1873	81	81
	CB16	91	89
D-M	4947	8.9	1.0
	1873	8.9	0.12
	CB16	7.9	0.9

[a] Incubation at 35°C.

In gastric juices, however, C-M toxin was more resistant than D-M toxin.

DISCUSSION

In the present study, different molecular-sized *C. botulinum* type C toxins, C-L (16S), and C-M (12S) toxins, were detected in both the culture supernatant and the cell extract of all the strains tested. C-L and C-M toxins were purified by essentially the same procedure as that adapted for purifying type F progenitor toxin except for the step of acid precipitation, in which ribonucleic acid was added to aid precipitation of the toxin (8). It has been reported that type D strain CB16 produced both L and M toxins, whereas type D strain 1873 produced M toxin only (13). In the present study both L and M toxins were purified from cultures of type D strains 4947, 1873, and CB16, and the different molecular-sized toxins were detected in the culture supernatant and the cell extract of type D strains 4947 and CB16 by ultracentrifugation, whereas a broad toxin peak was obtained with these materials of strain 1873. This may indicate that L toxin of type D strain 1873 might have been formed artificially during the purification procedures.

In sucrose density gradient ultracentrifugation at pH 6.0, C-L and C-M toxins sedimented to the same relative position as that of B-L and B-M toxins, respectively. It was also shown that C-L toxin at pH 7.0 or above resolved into toxic and nontoxic components in ultracentrifugation and so did C-M toxin in DEAE-Sephadex chromatography at pH 8.0. The dissociated toxic component of C-L and C-M toxins sedimented to the same position as that of type D derivative toxin (7S). These results indicate that type C toxin consists of toxic and nontoxic components that have the same molecular construction as those reported for type A, B, D, E, and F progenitor toxins (10, 11, 13, 15, 25).

The oral toxicities of different molecular-sized type C and D toxins showed a similar tendency to those of type A and B toxins; the larger the molecular size, the higher the oral toxicity. In Table 5, the oral toxicities and stabilities of the toxins of types A through F of different molecular sizes are summarized. B-L toxin purified from strain Okra showed an exceptionally high toxicity to mice; the oral toxicities of both C-L and D-L toxins were also high, being close to that of B-L toxin. Such high oral toxicities always reflect the high resistance of these toxins to gastric as well as intestinal juices. The ratio of the oral toxicity to the i.p. toxicity shown in Table 5 indicates the quantity of toxin protein in micrograms equivalent to 1 oral LD_{50} of each

TABLE 5. *Oral toxicities and stabilities of C. botulinum toxins of types A through F*

Toxin type	Toxicity		Ratio[d]	Stability[a] in	
	i.p.[b]	Oral[c]		Gastric juice[e]	Intestinal juice[f]
A-L	4.5×10^7	2.2×10^6	49		
A-M	8.5×10^7	3.6×10^6	42	<0.01	10
B-L[g]	4.5×10^7	1.5×10^3	0.03	29	88
B-M[g]	9.3×10^7	1.1×10^6	12	<0.01	6.2
C-L[h]	5.9×10^6	5.3×10^3	0.18	79	79
C-M[h]	1.5×10^7	1.6×10^5	11	8.6	<0.02
D-L[g,h]	2.2×10^6	6.2×10^4	0.28	93	77
D-M[g,h]	7.5×10^6	3.7×10^5	0.50	3.5	0.7
E[g,i]	1.0×10^7	3.7×10^5	37	0.7	39
F	2.1×10^7	1.1×10^6	52	<0.01	3.8

[a] Remaining toxicity (percent) after ination.
[b] i.p. LD_{50}/mg of protein.
[c] In number of i.p. LD_{50}.
[d] (Oral/i.p.) $\times 10^{-3}$.
[e] Incubated for 40 min at 35°C.
[f] Incubated for 180 min at 35°C.
[g] Toxicity after activation with trypsin.
[h] Figures given for L and M toxins of types C and D are the average of three strains of each type obtained in the present studies.
[i] G. Sakaguchi and S. Sakaguchi (21).

toxin. Since the molecular sizes of the L toxins and the M toxins are approximately the same for the different *C. botulinum* types, the ratios of the L toxin and M toxin also denote the relative mouse oral toxicity per molecule of L and M toxins. The ratios of B-L, C-L, and D-L toxins were lower than those of the others, including M toxins, showing higher oral toxicities to mice than the others. D-M toxin was the highest in the oral toxicity among M toxins of the six types. Table 5 shows also that C-M and D-M toxins (12S) were more stable in gastric juice than in intestinal juice; to the contrary A-M, B-M, E, and F toxins (10–12S) were more stable in intestinal juice. Such differences in the stability of botulinum toxins in the digestive juice may reflect different molecular configurations or conformations of these toxins.

Oral toxicities of botulinum toxins of different types to the same animal species and those of the same type to various animal species have been investigated. It was reported by Quortrup and Gorham (18) that oral administration of 5×10^7 mouse i.p. minimal lethal doses (MLD) of type C toxin did not kill foxes. Yndestad et al. (28) also reported extremely high resistance of foxes to type C toxin, 5×10^6 mouse i.p. MLD being not fatal, while other workers reported that an oral MLD of type C toxin to foxes was 10^4 mouse i.p. MLD (24). The oral lethal dose of type C toxin to minks has been reported to be in a wide range from 10^3 to 10^5 mouse i.p. MLD; 10^3 MLD according to Wagenaar et al. (29); 5×10^3 MLD according to Tjaberg and Skulberg (27); 10^5 MLD according to Quortrup and Gorham (18); and 10^5 MLD according to Karashimada et al. (9). Since C-L toxin is 30 times more potent in oral toxicity than C-M toxin as demonstrated in the present study, such diversity in oral toxicity of type C toxin to the same animal species may have possibly been due to the difference in the ratio of C-L to C-M toxin contents of the toxin preparations used by individual authors.

C. botulinum types C and D have exclusively been involved in animal botulism; a few outbreaks of human botulism caused by type C or D toxin have been reported. It has been demonstrated that the toxicity of types C and D is governed by specific bacteriophages and that the toxigenic strain could be converted into a nontoxigenic one and vice versa (2, 3, 6, 7). However, the relationship between toxigenic bacteriophages and the host cells, *C. botulinum* types C and D, was quite unstable probably due to pseudolysogeny (2, 3). This indicates that the toxigenicity of these types in nature may easily be lost under environmental conditions. It was suggested that the alteration of environmental factors, such as temperature, pH, salt concentration, etc., might influence the probability of lysis in lysogenic bacteria and that the balance between the lysogenic and lytic cycles may be the probable cause for variation in toxin production (5). In addition, it was noticed that toxigenic strains of *C. botulinum* type C require more strictly anaerobic conditions to grow and produce the toxin than other types (23, 24). Whether C-L and D-L toxins are produced in various types of food remains to be examined; the possibility may exist that human botulism could be caused by type C and D toxins if food containing large quantities of C-L or D-L toxin is ingested.

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Purification and Characterization of Two Components of Botulinum C_2 Toxin

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Two dissimilar proteins, designated as components I and II, of botulinum C_2 toxin elaborated by strain 92-13 were purified to a homogeneous state. The molecular weights determined by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II. Whereas each component showed no or feeble toxicity even after being treated with trypsin, the toxicity was elicited when these two components were mixed and trypsinized. The toxicity of the mixture of components I and II at a ratio of 1:2.5 on a protein basis was 2.2×10^4 mouse intraperitoneal 50% lethal doses per mg of protein and increased by 2,000 times or more when treated with trypsin. These results indicate that the molecular characteristics of botulinum C_2 toxin differ from those of the toxin of *Clostridium botulinum* types A through F in that C_2 toxin is constructed with two separate protein components, which are not covalently held together, and that its toxicity is elicited by cooperation of the two components.

Clostridium botulinum type C and D strains produce three antigenically different toxins, C_1, C_2, and D. The C_2 toxin is produced as a protoxin by certain strains of *C. botulinum* types C and D and those cured of thier prophages (3, 5, 12), so that toxicity can be demonstrated only after treatment with trypsin (3, 6). It has been reported that the trypsinized or endogenously nicked toxin molecules of *C. botulinum* types A through F are composed of two polypeptide chains (1, 10, 13, 18) which can be dissociated by treatment with a reducing agent in the presence of a detergent (8, 19). However, the association of these two chains is essential for eliciting the toxicity (19). In a previous paper, we reported that C_2 toxin consists of two cooperative components which are resolved by ion-exchange chromatography or gel filtration (4). These two components individually show very feeble toxicity even after treatment with trypsin, but full toxicity can be attained simply by mixing these two components. This suggests that the molecular construction of botulinum C_2 toxin differs from that of the toxin of *C. botulinum* types A through F. In the present paper, we describe the purification and characterization of these two components of C_2 toxin and compare the molecular construction of C_2 toxin with those of the other botulinum toxins.

MATERIALS AND METHODS

Bacterial strain and toxin production. Strain 92-13, resembling *C. botulinum* type C but producing only C_2 toxin (11), was provided by S. Nakamura, Kanazawa University, Kanazawa, Japan. Medium for toxin production was the chopped-meat medium described previously (4). A 1-ml inoculum containing 10^9 refractile spores was inoculated into 5,000 ml of the medium in a flat-bottomed spherical flask, and the culture was incubated for 2 days at 37°C.

Activation and toxicity assay of C_2 toxin. Tryptic activation was carried out in 0.05 M phosphate buffer, pH 8.0, for 30 min at 35°C unless otherwise indicated. Purified components I and II and C_2 toxin were activated at a toxin-to-trypsin ratio of 2:1 on a protein basis. Crude materials were treated with trypsin at a final concentration of 200 μg/ml. Trypsinization was terminated by adding an amount of soybean trypsin inhibitor that was twice the weight of trypsin. The toxicity in intraperitoneal mean lethal doses was determined in mice by the time-to-death method (4). When necessary, serial twofold dilutions of each sample were made in 0.05 M phosphate buffer, pH 8.0, containing 0.1% gelatin, and 0.1 ml was injected intraperitoneally or intravenously into separate groups of four mice. The 50% lethal dose was calculated from deaths within 4 days by the method of Reed and Muench (15).

Protein determination. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Electrophoresis in a 7.5% polyacrylamide gel at pH 4.0 was carried out by the method of Reisfeld et al. (16). Neutral red was used as a marker dye to measure relative mobility. Sodium dodecyl sulfate gel electrophoresis and molecular weight determinations were performed in a 6.0% polyacrylamide gel by the method of Dunker and Rueckert (2). The molecular weight was determined in sodium dodecyl sulfate gel electrophoresis from the mobility rate relative to that of α-chymotrypsinogen A (molecular weight = 25,000) by using the following protein standards: gamma globulin (molecular weight = 150,000), phosphorylase a (molec-

ular weight = 94,000), bovine serum albumin (molecular weight = 66,000), and ovalbumin (molecular weight = 45,000).

Anti-component I and II sera. Antiserum specific for each component was prepared. Purified component at 200 μg/ml was detoxified by dialysis against 0.05 M phosphate buffer, pH 8.0, containing 0.4% Formalin for 48 h at 30°C. A 0.5-ml (100 μg) portion of a toxoid was emulsified in an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and was injected subcutaneously into a rabbit. After 4 weeks, a 0.5-ml (100-μg) portion each of the untreated and homologous components was injected subcutaneously. The animals were bled after 2 weeks.

Neutralization test and immunodiffusion. A mixture of antiserum and toxin was held at room temperature (20 to 25°C) for 30 min and injected intravenously into mice to determine toxicity not neutralized. The agar gel double-diffusion test was performed by the method reported previously (7).

Purification of botulinum type C and D toxins. Botulinum type C and D progenitor toxins were purified from the culture of *C. botulinum* type C strain CB19 and type D strain CB16, respectively (10, 14).

Chemicals. Phosphorylase a and ovalbumin (Pentex Inc., Kankakee, Ill.), bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill.), gamma globulin (Schwarz/Mann, Orangeburg, N.Y.), and α-chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Soybean trypsin inhibitor was the product of Worthington Biochemicals Corp., Freehold, N.J.; trypsin (type III, twice crystallized) was from Sigma Chemical Co.

RESULTS

Purification of C_2 toxin. All procedures were performed at 4°C unless otherwise stated.

Step 1: precipitation with ammonium sulfate. Solid $(NH_4)_2SO_4$ was added to the whole culture to 55% saturation (380 g/liter), and the mixture was adjusted to pH 7.5 with 4 N NaOH. The precipitate formed by standing the mixture overnight was collected by centrifugation for 20 min at 4,650 × *g*. The precipitate from a 5-liter culture was suspended in 80 ml of 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, and the suspension was centrifuged for 20 min at 4,650 × *g*. This extraction procedure was repeated, and the supernatants were combined.

Step 2: acid precipitation. The supernatant was dialyzed against 3 liters of 0.05 M acetate buffer, pH 4.5, for 24 h. The precipitate formed during dialysis was collected by centrifugation for 10 min at 8,600 × *g* and extracted twice with 50 ml of acetate buffer containing 0.5 M NaCl. The toxic extract was dialyzed against 0.05 M Tris-hydrochloride, pH 7.5.

Step 3: diethylaminoethyl Sephadex chromatography. The precipitate formed during dialysis was removed by centrifugation for 10 min at 8,600 × *g*. The supernatant was applied to a column of diethylaminoethyl Sephadex A-50 (4 by 24 cm) equilibrated with the Tris-hydrochloride buffer. After sample application, the column was washed with 700 ml of the same buffer and then eluted with 1,000 ml of 0.3 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 7.5. The eluted fractions were pooled, concentrated to 60 ml by ultrafiltration with a PM-30 membrane, and dialyzed against 0.01 M acetate buffer, pH 6.0.

Step 4: carboxymethyl Sephadex chromatography. The dialyzed fraction was applied to a column of carboxymethyl Sephadex C-50 (2 by 10 cm) equilibrated with 0.01 M acetate buffer, pH 6.0. The column was washed with 60 ml of the buffer and eluted with 150 ml of the same buffer containing 0.3 M NaCl (Fig. 1). Each of the nonadsorbed and the eluate fractions had a toxicity of less than 1% of that applied. However, most toxicity was recovered when the two fractions were combined. Therefore, the nonadsorbed and the eluate fractions were designated as components I and II, respectively. These two fractions were concentrated separately by ultrafiltration through a PM-30 membrane.

Step 5: gel filtration on Sephadex G-100. The concentrated components were applied to separate Sephadex G-100 columns (2.5 × 95 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, and eluted with the same buffer (Fig. 2); the component I was rechromatographed on the Sephadex G-100 column. High toxicity was demonstrated when components I and II were mixed and trypsinized, whereas each component showed a very low toxicity.

The overall purification is summarized in Table 1. The recovery of toxicity was 20% of that in the culture as determined by the toxicity obtained by trypsinizing a mixutre that was made by combining components I and II in the ratio of their concentrate volumes. The degree of the purification on a toxicity basis was 95-fold relative to the toxic preparation obtained by precipitation with $(NH_4)_2SO_4$.

Polyacrylamide gel electrophoresis. Purified components I and II each showed a single band in disc electrophoresis at pH 4.0. Electrophoretic mobilities relative to the marker dye were 0.51 and 0.42, respectively (Fig. 3). In sodium dodecyl sulfate gel electrophoresis, the purified components before and after treatment with 2-mercaptoethanol each showed a single band (Fig. 4). The molecular weights estimated by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II.

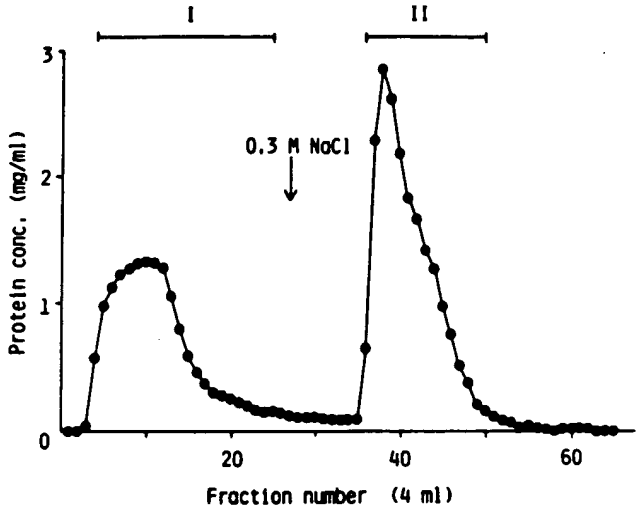

FIG. 1. *Carboxymethyl Sephadex chromatography of C_2 toxin. The toxin fractions obtained by diethylaminoethyl Sephadex chromatography were applied to a carboxymethyl Sephadex C-50 column, and 4-ml fractions were collected. The unabsorbed fractions were pooled as component I, and the eluate fractions were pooled as component II (horizontal bars).*

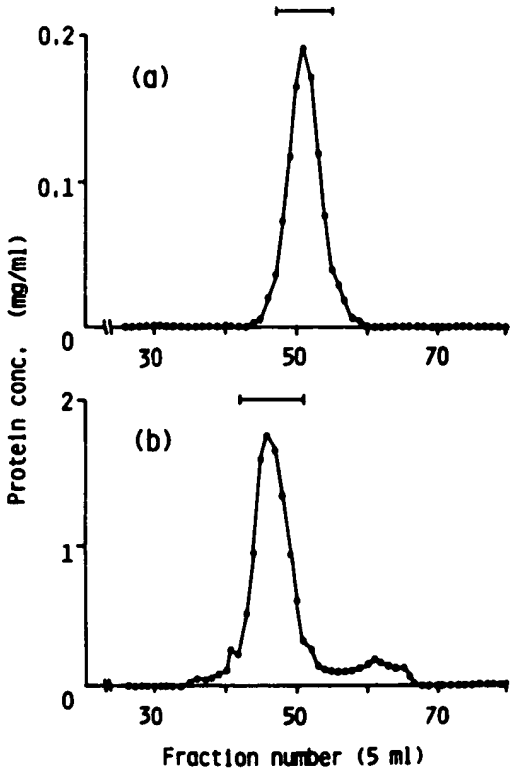

FIG. 2. *Gel filtration of components I and II on Sephadex G-100. Components I and II from carboxymethyl Sephadex chromatography were applied separately to a Sephadex G-100 column. The fractions indicated by the horizontal bars were pooled. (a) Elution pattern of the second gel filtration of component I; (b) elution pattern of gel filtration of component II.*

Toxicities. The toxicities of C_2 toxin were studied in a mixture made by adding various amounts of component I to a fixed amount of component II and by treating with trypsin (Fig. 5). The maximum toxicity was obtained when the ratio of component I to component II was from 1:2.0 to 1:2.5. Mixtures of these ratios increased 2,000-fold or more in toxicity when treated with trypsin. From these results, C_2 toxin was defined in the present experiments as a mixture of components I and II at a ratio of 1:2.5 on a protein basis. Component II showed a very low toxicity after treatment with trypsin, whereas component I before or after trypsin treatment was not lethal to mice when 100 μg was injected intravenously (Table 2).

The toxicities of different combinations of trypsinized and untrypsinized component I or II were examined. A mixture of trypsinized component I and untrypsinized component II was not lethal to mice, whereas that of untrypsinized component I and trypsinized component II showed 82% of the toxicity of trypsinized C_2 toxin (Table 3).

The toxicity of purified C_2 toxin to mice by the intravenous route was higher than that by the intraperitoneal route; one 50% lethal dose required 5.4 ng of protein by the intravenous route and 49 ng by the intraperitoneal route.

Activation conditions. The purified C_2 toxin

TABLE 1. *Purification of components I and II of botulinum C_2 toxin*

Step	Volume (ml)	Protein (mg)	Potential toxicity ($\times 10^{-4}$ i.p. LD_{50})[a]	Recovery (%)[b]	Specific toxicity ($\times 10^{-2}$ i.p. LD_{50}/mg of protein)[a]
Whole culture	5,000		472	100	
Ammonium sulfate precipitation	100	24,600	460	98	1.9
Extract of ammonium sulfate precipitate	160	17,500	451	96	2.6
Extract of acid precipitate	100	4,570	394	84	8.6
Diethylaminoethyl effluent	60	686	400	85	58.3
Carboxymethyl Sephadex					
Nonabsorbed (component I)	120	82	—[c]	NS[d]	
Eluate (component II)	150	166	3.3	0.7	2.0
Mixture of I and II[e]			320	68	129.0
Sephadex G-100					
Component I	45	4.8	*[f]	NS[d]	
Component II	50	48.5	3.1	0.7	6.4
Mixture of I and II[e]			96	20	180.1

[a] i.p. LD_{50}, Intraperitoneal 50% lethal dose.
[b] Toxicity of whole culture was taken as 100%.
[c] —, Mice survived for 100 min but not 6 h after intravenous injection of 4 μg of trypsinized protein.
[d] NS, Not significant.
[e] Toxicity after trypsinizing a mixture made of components I and II in a ratio corresponding to volumes of the two fractions.
[f] * Mice survived after intravenous injection of 4 μg of trypsinized protein.

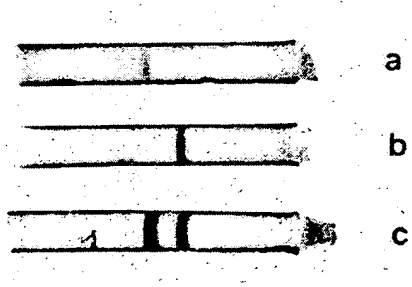

FIG. 3. *Polyacrylamide gel electrophoresis of purified components I and II. A 10-μg portion of purified component I or II was applied to a gel column and electrophoresed for 210 min at 3 mA per column. (a) Component I, (b) component II, and (c) a mixture of I and II.*

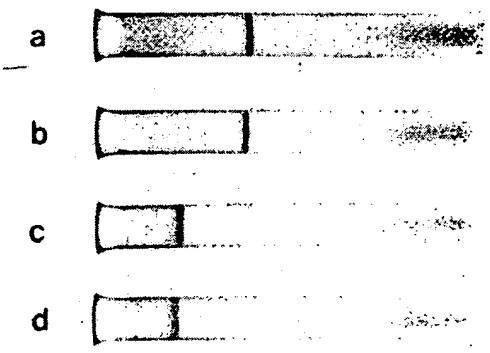

FIG. 4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of components I and II. A 5-μg portion of purified component I or II was applied on a gel column (4 by 70 mm) and electrophoresed for 240 min at 6 mA per gel. (a) Component I before and (b) after reduction; (c) component II before and (d) after reduction.*

was treated with trypsin at different pH's for 30 min at 35°C, and the toxicities were determined. The optimum condition of activation was pH 8.0. Maximum activation of C_2 toxin was attained by incubation for 30 min at a trypsin-to-toxin ratio of 1:2 (Fig. 6). Toxicity persisted on the same level for 180 min.

Immunodiffusion and neutralization tests. In agar gel double-diffusion tests, purified components I and II each gave a single precipitin line against a mixture of rabbit anti-component I and II sera that crossed each other. The C_2 toxin, a mixture of components I and II, formed two precipitin lines, of which one fused in line of identity with the line of component I, and the other fused with that of component II (Fig. 7). Neither anti-component I nor II serum formed a precipitin line with 100 μg of the progenitor toxins of *C. botulinum* types C or D. A 10-μl

portion of anti-component I or II serum completely neutralized 20 mouse intraperitoneal 50% lethal doses of activated C_2 toxin but did not neutralize the same amount of type C or D toxin.

DISCUSSION

The evidence of the present study shows that the two dissimilar protein components, designated as components I and II, are requisite for the lethality of C_2 toxin in mice; a markedly higher toxicity was obtained when components I and II were mixed and treated with trypsin, whereas the components individually show a very low toxicity even after trypsinization. In a previous paper, we reported that these two components in culture supernatant can be separated by ion-exchange chromatography or gel filtration without treatment with detergent, reducing agent, or protease (4), indicating that components I and II exist as separate forms in culture fluid. This was confirmed in the present study by the successful purification of components I and II without such treatments. It has been reported that the trypsinized or endogenously nicked toxic components of types A through F progenitor toxins consist of two fragments with approximate molecular weights of 100,000 and 50,000 linked together with a disulfide bridge(s) (1, 10, 13, 17, 18). The fragments of type B and C toxins can be resolved by chromatography only when treated with both a detergent and a reducing agent (8, 19). The toxicity is lost concomitant to dissociation; it is restored on reassociation of these two fragments by removing

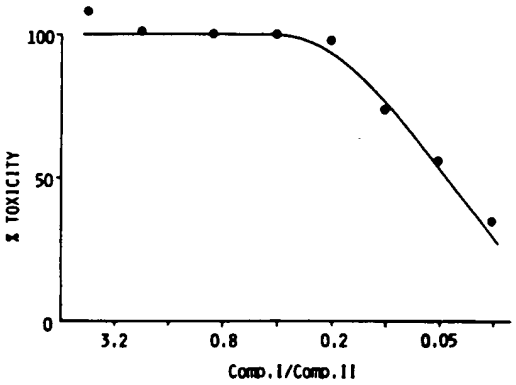

FIG. 5. *Toxicity of C_2 toxin in mixture made with various ratios of components I and II. A 0.25-ml portion of component I containing 2 to 400 μg of protein per ml was added to 0.25 ml of component II containing 80 μg of protein per ml. Mixtures were treated with trypsin at 200 μg/ml. Toxicity in mixture made with the ratio of 0.4 (1:2.5 on a protein basis) was taken as 100%.*

TABLE 2. *Toxicities of components I and II and C_2 toxin before and after trypsinization*

Toxin	Toxicity (i.p. LD_{50}/100 μg of protein)[a]	
	Trypsinized	Untrypsinized
Component I	•[b]	•
Component II	50	•
C_2 toxin[c]	2,200	—[d]

[a] i.p. LD_{50}, Intraperitoneal 50% lethal dose.
[b] •, Mice survived after intravenous injection.
[c] Components I and II were mixed at the ratio of 1:2.5 on a protein basis.
[d] —, Mice survived for 100 min but not 6 h.

TABLE 3. *Toxicity in mixtures made with different combinations of trypsinized and untrypsinized components I and II*[a]

Mixture	Toxicity (i.p. LD_{50}/ml)[b]	Ratio[c]
T-I + T-II[d]	17.9	1.0
T-I + UT-II[e]	—[f]	
UT-I + T-II[g]	14.6	0.82
UT-I + UT-II[h]	—[f]	

[a] Equal volumes of trypsinized (T) and untrypsinized (UT) component I (2.3 μg/ml) and component II (5.8 μg/ml) were mixed, and the toxicity was determined. Trypsinization of each component was carried out as described in the text.
[b] i.p. LD_{50}, Intraperitoneal 50% lethal dose.
[c] Toxicity of T-I + T-II taken as 1.0.
[d] Both components trypsinized.
[e] Trypsinized component I plus untrypsinized component II.
[f] —, Mice survived for 100 min but not 6 h.
[g] Untrypsinized component I plus trypsinized component II.
[h] Neither component trypsinized.

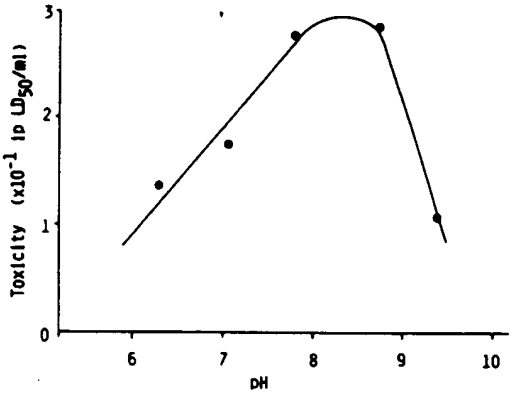

FIG. 6. *Activation of C_2 toxin with trypsin at different pH values. A 0.25-ml portion of C_2 toxin containing 5 μg of component I and 12.5 μg of component II was mixed with an equal volume of each of the buffer solutions at different pH values containing 400 μg of trypsin. The buffers used were 0.05 M phosphate buffer, pH 6.3 and 7.1, and 0.1 M Tris-hydrochloride, pH 7.9, 8.8, and 9.4.*

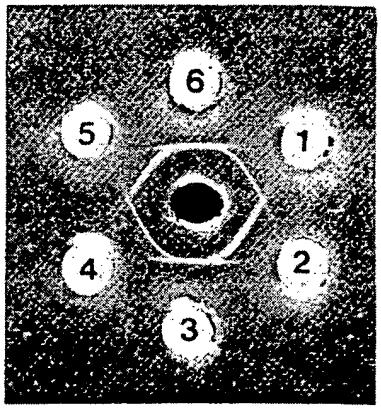

FIG. 7. *Agar gel double-diffusion tests with purified components I and II. Center well, a mixture of equal volumes of anti-component I and anti-component II sera; lateral wells (1) and (4), component I, 55 μg/ml; (2) and (5), component II, 100 μg/ml; (3) and (6), C_2 toxin, 155 μg/ml.*

the detergent and the reducing agent (19). The toxicity of botulinum C_2 toxin, however, was regenerated simply by mixing components I and II, indicating that the in vitro binding of component I and II molecules is not required for toxicity. The molecular construction of C_2 toxin is entirely different from that of the toxic component of progenitor toxins of *C. botulinum* types A through F in that its components are not covalently held together, so the components exist naturally as separate molecules.

Gel filtration, polyacrylamide gel electrophoresis, and sucrose density ultracentrifugation were tried in attempts to see whether purified components I and II of C_2 toxin form a complex in vitro. But all attempts to demonstrate the complex failed. As we reported previously, the separation of components I and II of botulinum C_2 toxin was observed with 1- or 2-day cultures of *C. botulinum* types C and D and strains producing only C_2 toxin (4). It was also demonstrated in sodium dodecyl sulfate gel electrophoresis that components I and II each gave a single band even after reduction. These results indicate that C_2 toxin intrinsically constitutes two separate polypeptides, although this does not rule out the possibility that these two components form a complex at the site where biological activity occurs.

In agar gel diffusion, purified components I and II each formed a single precipitin line; they crossed each other. This shows that components I and II are immunologically distinct protein molecules. Anti-component I or II serum gave no precipitin line with the progenitor toxin of *C. botulinum* types C and D and did not neutralize both of these toxins. This indicates that the components of C_2 toxin have no antigenic relation with these botulinum toxin types.

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Botulinum G Neurotoxin Cleaves VAMP/Synaptobrevin at a Single Ala-Ala Peptide Bond*

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Similarly to other serotypes, botulinum neurotoxin serotype G (BoNT/G) contains the zinc binding motif of zinc endopeptidases. Highly purified preparations of BoNT/G show a zinc-dependent protease activity specific for VAMP/synaptobrevin, a membrane protein of synaptic vesicles. The two neuronal VAMP isoforms are cleaved with similar rates at one Ala-Ala peptide bond present in the same region, out of the several such peptide bonds present in their sequences. This site of cleavage is unique among the eight clostridial neurotoxins. VAMP proteolysis is displayed only after reduction of the single interchain disulfide bond present in the toxin, and it is inhibited by EDTA, o-phenanthroline and captopril.

Botulinum neurotoxins (BoNT)[1] are responsible for all the clinical symptoms of botulism. In fact, the flaccid paralysis of botulism results from the toxin-induced block of acetylcholine release at the neuromuscular junction (1, 2). BoNT are produced by bacteria of the genus *Clostridium* in seven different serotypes: A, B, C, D, E, F, and G. BoNT/G was the last BoNT serotype discovered and is produced by a *Clostridium botulinum* originally isolated from the soil (3). It was later reported to be found in necropsy in humans who had died suddenly from an unidentified cause (4).

The toxin is synthesized as a single polypeptide chain of 150 kDa, which is cleaved by proteases at a single site with the generation of two disulfide-linked chains. The heavy chain (H, 100 kDa) is responsible for neurospecific binding and membrane translocation, while the light chain (L, 50 kDa) blocks neuroexocytosis (5, 6).

Recently, BoNT/A, /B, /E, and/F, as well as tetanus neurotoxin (TeNT), were shown to contain one atom of zinc bound to the zinc binding motif of zinc endopeptidases present in the central part of their L chains (7–10). The sequence of serotype G of BoNT has been reported very recently and shows the presence of the same His-Glu-Xaa-Xaa-His motif (11).

The specific proteolytic target of TeNT, BoNT/B, BoNT/D, and BoNT/F was demonstrated to be VAMP/synaptobrevin (VAMP), a protein involved in docking and fusion of small synaptic vesicles with the presynaptic membrane (7, 9, 12–16). VAMP was first identified in the *Torpedo* electric organ (17) and later cloned from rat, bovine, human, *Drosophila*, and yeast (18–21). Rat brain VAMP consists of two isoforms, termed VAMP-1 and VAMP-2, of 118 and 116 amino acids, respectively, which differ in the 33-residue-long amino-terminal part (18). Recently, an isoform of VAMP with a shorter amino-terminal domain, termed cellubrevin, has been identified in non-neuronal cells and shown to be associated with endosomes (22). The central region (residues 33–96) of VAMP is highly conserved among species, and it is also very similar in the three VAMP isoforms. VAMP is anchored to vesicles by a carboxyl-terminal hydrophobic tail, while the bulk of the molecule is exposed to the cytosol.

Both TeNT and BoNT/B hydrolyze specifically the peptide bond between Gln-76 and Phe-77 of rat VAMP-2, as shown in Fig. 4 (12). Rat VAMP-1 is toxin-resistant, most likely because of a replacement of Val for Gln at the cleavage site (23). BoNT/D and /F cleave both VAMP-2 and -1 isoforms at single Lys-Leu and Gln-Lys peptide bonds, respectively (see Fig. 4). No other substrate was found in other membrane or soluble fractions of the nervous tissue. In contrast, BoNT/A and /E cleave specifically SNAP-25 at two different sites (13, 24–26), and BoNT/C cleaves syntaxin (27). Both SNAP-25 and syntaxin are membrane proteins localized on the presynaptic membrane and with the majority of the molecule exposed to the cytosol (28, 29).

The only remaining BoNT serotype whose target was still to be determined was G. Here we report that both in synaptosomes and isolated small synaptic vesicles BoNT/G recognizes specifically and cleaves both rat VAMP-1 and -2 at a single Ala-Ala peptide bond, out of the four such bonds present in the sequence.

MATERIALS AND METHODS

Proteins and Chemicals—TeNT and BoNT/A, /B, /C, /D, and /F were obtained as described previously (7–9, 13). *C. botulinum* type G strain 89 was grown for toxin production in TPPGY medium (30) for 6 days at 30 °C under 10% CO_2, 90% N_2. Toxins were precipitated by the addition of 0.2 g/liter yeast RNA (Sigma) and by lowering the pH to 3.4 with 3 N sulfuric acid. The precipitate was extracted at room temperature with 0.2 M sodium phosphate, 0.5 M NaCl, pH 7.0, and clarified by centrifugation. The supernatant was precipitated with $(NH_4)_2SO_4$ (60% of saturation), centrifuged, and dissolved in 50 mM sodium phosphate, pH 6.0. The toxin solution was then treated with 50 μg/ml RNase (Sigma) for 3 h at 37 °C, precipitated with 60% of $(NH_4)_2SO_4$. After dissolution in 50 mM sodium citrate, pH 5.5, the toxin was applied to a DEAE-Sephadex A-50 column, and the non-retained pool was precipitated with 60% of $(NH_4)_2SO_4$, solubilized in 20 mM sodium acetate, pH 6.0, and loaded onto a SP-Sephadex C-50 column. The toxin was eluted with a linear 0–0.5 M NaCl gradient, pooled, and, after dialysis against 20 mM sodium acetate, pH 6.0, loaded onto a *p*-aminophenyl-β-D-thiogalactopyranoside affinity column and eluted as described (30). Affinity-purified BoNT/G was further purified on a TSK G3000 SW column (0.75 × 60 cm, Phar-

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[1] The abbreviations used are: BoNT, botulinum neurotoxins; DTT, dithiothreitol; GST-VAMP/2, recombinant glutathione *S*-methyltransferase VAMP-2 fusion protein; SSV, small synaptic vesicles; VAMP-1, VAMP/synaptobrevin isoform 1; VAMP-2, VAMP/synaptobrevin isoform 2; TeNT, tetanus neurotoxin; PAGE, polyacrylamide gel electrophoresis.

macia Biotech Inc.) equilibrated in 100 mM sodium phosphate, pH 6.8. BoNT/G had a specific activity >10^7 MLD_{50}/mg of protein.

Captopril ([2*S*]-1-(3-mercapto-2-methylpropionyl)-1-proline) was from Squibb (Italy). Small synaptic vesicles were isolated from rat cerebral cortex, omitting the glass bead chromatography step (31) and, after addition of 100 μM phenylmethylsulfonyl fluoride and 2 μg/ml pepstatin (Sigma), used immediately. Recombinant glutathione *S*-methyltransferase VAMP-2 fusion protein (GST-VAMP/2) was obtained by inserting the 1–116 construct of VAMP-2 into *Sma*I and *Eco*RI sites of plasmid pGEX-KG (32) and transformed into the AB1899 strain of *Escherichia coli*. GST-fusion protein was purified by affinity chromatography on GSH-agarose matrix (Sigma), followed, when necessary, by ion exchange chromatography on Mono-Q (Pharmacia).

Antibodies—Rabbit polyclonal antisera against rat SNAP-25 and a mixture of rat brain VAMPs were prepared as reported (28, 33). A mouse monoclonal antibody against rat retina synaptophysin was purchased from Sigma, while the one specific for rat synaptotagmin was kindly provided by Dr. M. Popoli (University of Milan, Italy). Horse antiserum against BoNT serotype G was a kind gift of J. E. Brown (Fort Detrick, MD). Affinity-purified rabbit polyclonal antibodies anti-peptides 1–33 of rat VAMP-1 and -2 were a kind gift of Dr. O. Rossetto (University of Padua).

Proteolytic Activity of BoNT/G and Other BoNT Serotypes on Synaptosomes, Small Synaptic Vesicles, and Recombinant VAMP-2 Fusion Protein—50 μg of synaptosomes, isolated from rat brain cortex (13), were incubated with the different BoNT serotypes (50 nM) in 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, 0.8% 1-*O*-*n*-octyl-β-D-glucopyranoside, 20 mM HEPES-Na pH 7.4 for 4 h at 37 °C. 30 μg of SSV in 30 μl of 5 mM HEPES-Na, 0.3 M glycine, 0.15 M NaCl, 0.02% NaN_3 or 5 μg of GST-VAMP/2 in 30 μl of 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4 were treated for 120 min at 37 °C with a final concentration of 30 nM native BoNT/G or BoNT/G previously reduced with 10 mM dithiothreitol for 30 min at 37 °C. In some samples reduced toxin was preincubated for 30 min at 37 °C with different inhibitors (final concentrations: captopril, 2 mM; *o*-phenanthroline, 1 mM; EDTA, 1 mM). In other experiments, BoNT/G was mixed with horse polyclonal anti-BoNT/G antibodies and then treated with 100 μl of a 50% suspension of Protein A-agarose (ICN). After 2 h at 4 °C, the supernatant was recovered by centrifugation at 15,000 × *g* and reduced as described previously.

Some BoNT/G-treated SSV samples (35 μl) were diluted with 5 mM HEPES-Na, 10 mM EDTA to a final volume of 100 μl, overlaid on 10 mM HEPES-Na pH 7.4, 120 mM NaCl and centrifuged for 60 min at 350,000 × *g* in a TL-100 centrifuge (Beckman). Supernatants were precipitated with trichloroacetic acid and centrifuged for 15 min at 15,000 × *g*, and the resulting pellets were dissolved in 8% SDS, 10 mM Tris acetate, pH 8.2, 0.1 mM EDTA. Pellets derived from the TL-100 centrifugation were solubilized in the same buffer and boiled for 2 min. Time course measurements of VAMP proteolysis were performed as before (13).

Immunoblotting—Selected samples were transferred onto nitrocellulose as described elsewhere (13) and treated with anti-VAMP-1 or -2 specific antisera (1:500 dilution), anti-SNAP-25 (1:1,000), anti-syntaxin (1:200), anti-synaptophysin (1:1,000), or anti-synaptotagmin (1:1,000). VAMP-1 and VAMP-2 content was determined by specific anti-VAMP-1 and anti-VAMP-2 polyclonal antibodies (1:500 dilution). The primary antibodies were detected by immunostaining with an anti-rabbit (1:10,000 dilution, Boehringer Mannheim) or anti-mouse antibody conjugated with alkaline phosphatase (1:1,000, Sigma) (13). Immunoblots were quantified by scanning with a Shimadzu CS-630 densitometer.

Protein Sequencing—For sequence analysis, VAMP fragments were electroeluted from polyacrylamide gels, applied to ProSpin tubes (Applied Biosystems), and sequenced in a pulsed liquid Applied Biosystems model 477A protein sequencer.

RESULTS AND DISCUSSION

Two recent observations led us to test the possibility that BoNT/G might act as a specific protease. First, its sequence revealed that, like the other clostridial neurotoxins, BoNT/G has the zinc binding motif of zinc endopeptidases. Second, recent studies have shown that TeNT, BoNT/B, /D, and /F specifically cleave VAMP (7, 9, 12–14), BoNT/A and /E cleave SNAP-25 (13, 24–26), and BoNT/C cleaves syntaxin (27). To determine if BoNT/G might cleave any of these targets, permeabilized rat brain synaptosomes were mixed with BoNT/G and the amount of VAMP, SNAP-25, and syntaxin immunoreactivity was assayed by immunoblotting. Fig. 1 shows that BoNT/G caused a strong reduction in VAMP immunoreactivity but had no effect on SNAP-25, syntaxin, or the control SSV proteins synaptotagmin and synaptophysin.

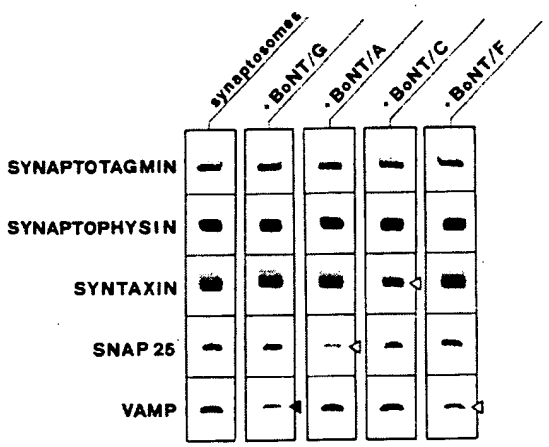

FIG. 1. **Effect of BoNT/G, /A, /C, and /F on the level of synaptotagmin, synaptophysin, SNAP-25, and VAMP on rat brain synaptosomes.** Rat brain synaptosomes were treated with either BoNT/G, /A, /C, or /F as detailed under "Materials and Methods," electrophoresed, and blotted onto nitrocellulose membranes. Different membranes were probed with a mouse anti-synaptotagmin or with anti-synaptophysin monoclonal IgG, rabbit anti-syntaxin, anti-SNAP-25, or anti-VAMP antisera and stained with the appropriate alkaline phosphatase-conjugated anti-IgG antibodies.

To determine the specificity of proteolytic activity, purified SSVs from rat brain cortex were incubated with BoNT/G. The only change in the SDS-PAGE protein profile of SSV is the disappearance of the VAMP band, having an apparent electrophoretic mobility of 18 kDa, and the appearance of two new bands of 13 and 6 kDa (F13 and F6). The size of the two BoNT/G-generated VAMP fragments differs from those produced by the other VAMP-specific neurotoxins. This result is indicative of a site of proteolytic cleavage, different from those of TeNT, BoNT/B, /D, and /F. No smaller peptides could be detected even in highly cross-linked polyacrylamide gels, and this suggests, together with the observation that the sum of the two fragments gives the size of the original protein, that VAMP is cleaved by BoNT/G at a single site. The comparison of the fourth and fifth lane of Fig. 2*A* shows that BoNT/G is proteolytically active only after reduction. This proteolytic activity is entirely due to the presence of BoNT/G in the sample, as demonstrated by the absence of any VAMP cleavage after incubation with a specific antiserum against BoNT/G. Fig. 2*A* also shows that BoNT/G activity was blocked by EDTA and *o*-phenanthroline, two general inhibitors of zinc proteases. BoNT/G proteolytic activity is almost completely inhibited by captopril, a widely used antihypertensive agent, which blocks specifically the angiotensin-converting enzyme, a well characterized zinc endopeptidase (34). Although captopril is a poor inhibitor of BoNT/G, this result suggests that it is possible to synthesize derivatives of higher affinity to be tested as therapeutic agents in botulism.

Fig. 2*B* shows that BoNT/G is able to cleave a recombinant GST-VAMP/2 *in vitro*. This protein is converted into two fragments of 36 and 6 kDa; the larger fragment corresponds to the glutathione *S*-methyltransferase fused with the amino-terminal end of VAMP-2, while the smaller fragment has an identical electrophoretic mobility to fragment F6 produced upon incubation of SSV. This indicates that the cleavage of VAMP does not require any additional factor present in SSV. Furthermore, the comparison of the mobility of F36 and F6 with those of the fragments generated by incubation of GST-VAMP/2 with different BoNTs confirms that BoNT/G cleaves at a site different

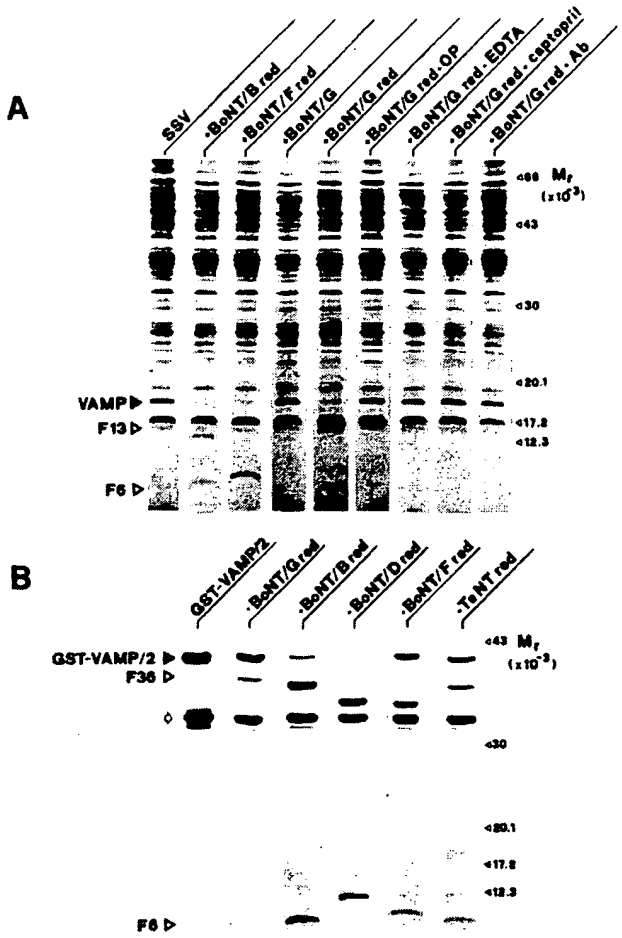

FIG. 2. **Botulinum neurotoxin type G proteolysis of small synaptic vesicles (*A*) and recombinant VAMP-2 (*B*).** *A*, Coomassie Blue-stained SDS-PAGE profile of SSV alone or after incubation with unreduced BoNT/G (*+BoNT/G*) or DTT-reduced BoNT/B (*+BoNT/B red*), DTT-reduced BoNT/F (*+BoNT/F red*), DTT-reduced BoNT/G (*+BoNT/G red*), or DTT-reduced BoNT/G in the presence of 1 mM *o*-phenanthroline (*+BoNT/G red + OP*), or 1 mM EDTA (*+BoNT/G red + EDTA*), or 2 mM captopril (*+BoNT/G red + captopril*). *BoNT/G red + Ab* refers to a sample in which BoNT/G had been pretreated with a horse anti-serotype G-specific antiserum (see "Materials and Methods"). The position of VAMP and of the BoNT/G-induced fragments (F13 and F6) are indicated by *closed* and *open triangles*, respectively. *B*, silver-stained SDS-PAGE profile of recombinant GST-VAMP/2 incubated alone or in the presence 50 nM DTT-reduced BoNT serotypes /G, /B, /D, /F, and TeNT. The F36 and F6 fragments, generated by BoNT/G, are indicated by *open triangles*. The *small arrow* indicates a GST-VAMP/2-truncated fusion protein.

from those of TeNT, BoNT/B, /D, and /F.

To determine the isoform specificity and relative cleavage rates of VAMPs, SSV were incubated with BoNT/G for different time periods and, after SDS-PAGE, transferred onto nitrocellulose membranes. The amount of VAMP-1 and -2 was determined by quantitative immunoblotting with isoform-specific antibodies raised in rabbits *versus* the amino-terminal part of the two VAMP isoforms and densitometry. Fig. 3 shows that both VAMP-1 and VAMP-2 are cut by BoNT/G with very similar apparent rates of hydrolysis. By contrast the rate of cleavage of VAMP-1 mediated by BoNT/B is much lower than that of VAMP-2, as expected on the basis of the previous findings (12).

To separate and isolate the two fragments, F13 and F6, after incubation with the BoNT/G, SSV were diluted, loaded on a low ionic strength solution, and ultracentrifuged. Electrophoresis of the supernatant and pellet fractions revealed that the larger

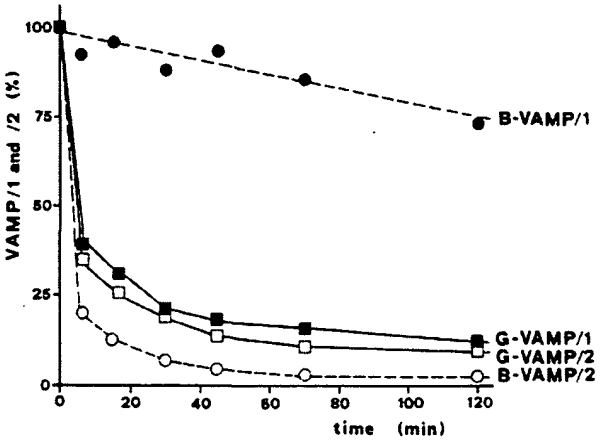

FIG. 3. **Time course of proteolysis of VAMP-1 and VAMP-2 on small synaptic vesicles by botulinum type B and G neurotoxins.** SSV samples were treated at 37 °C with DTT-reduced BoNT/G (30 nM; *squares*) or BoNT/B (5 nM; *circles*) for different periods of time, electrophoresed, and blotted. The nitrocellulose membranes were probed with rabbit affinity-purified anti-VAMP-1 (*closed symbols*) and anti-VAMP-2 (*open symbols*) antibodies, detected as in Fig. 1 and quantified by densitometry. Results are expressed as a percentage of the initial VAMP content. Each time point was processed in duplicate, and final data are averages of three independent experiments; S.D. bars are omitted for clarity and never exceeded 9%.

F13 fragment was released to the supernatant, while the smaller F6 fragment remained bound to the SSV membranes in the pellet. The release from the neurotransmitter-containing vesicles of a major portion of the cytosolic domain of VAMP is expected to prevent the assembly of a functional 20 S neuroexocytosis apparatus, thus accounting for the persistent blockade of neuroexocytosis caused by BoNT/G (4).

After electroelution, the two fragments were sequenced. F13 gave no sequence, in agreement with the fact that the amino terminus of VAMP is acetylated (15). As reported in Fig. 4, F6 gave the sequence AKLKRKYWWKNLKMMI consistent with a cleavage of the Ala^{81}-Ala^{82} peptide bond of VAMP-2 and the corresponding Ala^{83}-Ala^{84} bond of VAMP-1. The Cys and Met residues present in the sequence are lost under the present conditions of sequencing. The VAMP region cleaved by BoNT/G is absolutely conserved among animal species and isoforms. The same site was cleaved by BoNT/G on recombinant VAMP-2.

Based on comparison of the known cleavage sites of clostridial neurotoxins, the present identification of an Ala-Ala peptide bond as the site of cleavage of BoNT/G was unexpected. In fact, TeNT and BoNT/A, /B, and /F cleave peptide bonds with a Gln on the P_1 site (the amino-terminal site of the cleaved peptide bond), while BoNT/D and /E recognize positively charged residues in the same position. On the P_1' (the carboxyl-terminal site of the cleaved peptide bond) these proteinases recognize either a positive residue (BoNT/A and/F) or a bulky hydrophobic residue (TeNT, BoNT/B, /D, /E). It is also noteworthy that BoNT/G cleaves only one of the three Ala-Ala peptide bonds present in the sequence of VAMP-2 and one of the two present in VAMP-1. In this sense, BoNT/G resembles the other clostridial neurotoxins (14, 35) and differs from most proteases in that it appears to recognize the tertiary structure of the target rather than the sequence of the peptide bond to be cleaved. The region of VAMP containing the Ala-Ala peptide bond hydrolyzed by BoNT/G is predicted by computer programs to be highly flexible.

In conclusion, this paper provides evidence that the molecular pathogenesis of type G botulism derives from a zinc-dependent endopeptidase activity of BoNT/G specifically directed at the Ala-Ala peptide bond present in the carboxyl-terminal part of both VAMP-1 and VAMP-2. This finding opens the way for a

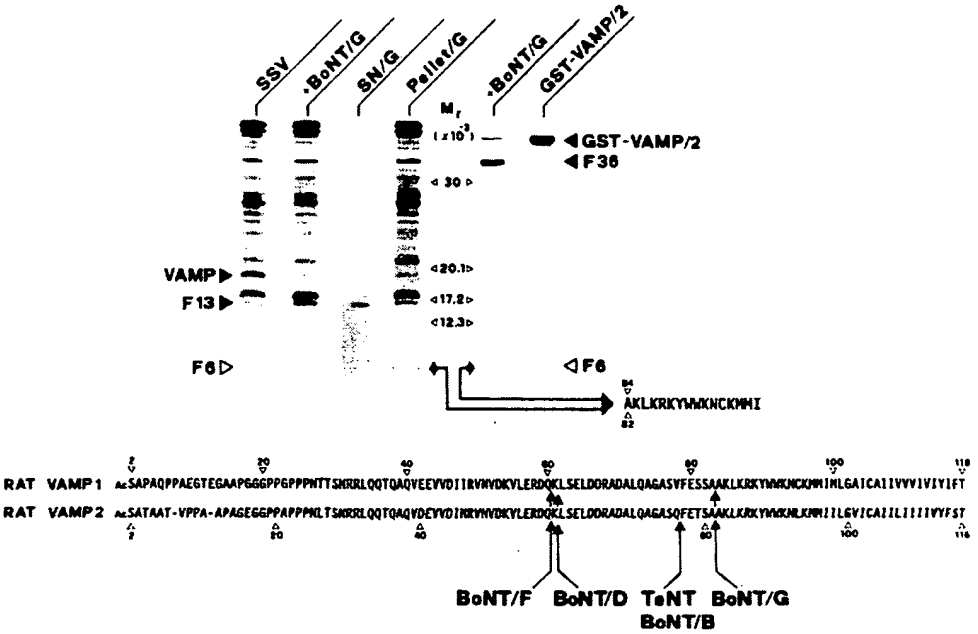

FIG. 4. **Sequences of VAMP fragments generated by BoNT/G proteolysis.** SSV and purified recombinant GST-VAMP/2 were incubated at 37 °C alone or in the presence of BoNT/G. Half of the SSV BoNT/G-treated sample was solubilized in SDS-electrophoresis buffer (*BoNT/G*), while the other half was ultracentrifuged. Pellet (*Pellet/G*) and supernatant (*SN/G*) fractions were then electrophoresed, and the BoNT/G-induced F13 and F6 VAMP fragments were electroeluted and sequenced. The F13 soluble fragment gave no sequence, while both the F6 fragments derived from SSV and recombinant VAMP-2 cleavage provided the sequence shown in *larger letters*. VAMP-1 and -2 sequences are reported in the *lower panel* for comparison, together with the deduced cleavage sites of BoNT/F (9), BoNT/D (13), BoNT/B, and TeNT (12).

search of suitable inhibitors and provides further evidence for an as yet unknown but fundamental role of VAMP in exocytosis. It can be speculated that the existence of four different sites of VAMP attack by different neurotoxins produced by *Clostridium* spp. may be related to the advantage for the bacterium to overcome the effect of mutating the site of toxin cleavage. We have recently proposed that the toxin resistance of neuronal rat and chicken VAMP-1 may account for the low sensitivity of chickens and rats to tetanus and BoNT/B botulism (23).

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The Theory and Practice of Industrial Pharmacy

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formation relative to the influence of temperature on solubility should be generated. As a rule, a solution should be designed in which the solubility of the solute is not exceeded even at temperatures as low as 4°C.

The approach used when the required concentration of drug exceeds the aforementioned solubility criteria depends on the chemical nature of the drug and the tvpe of product desired.

pH. A large number of modern chemotherapeutic agents are either weak acids or weak bases. The solubility of these agents can be markedly influenced by the pH of their environment. Through application of the law of mass action, the solubility of weakly acidic or basic drugs can be predicted, as a function of pH, with a considerable degree of accuracy. Consider, for example, the reactions involved in the dissolution of a weakly acidic drug, DH:

$$\text{DH (solid)} \rightleftharpoons \text{DH (solution)} \qquad (1)$$

where DH (solution) is equal to the solubility of the undissociated acid in moles per liter and is a constant generally referred to as Ks.

The undissociated acid is also in equilibrium with its dissociation products:

$$\text{DH (solution)} \rightleftharpoons D^- + H^+$$

$$Ka = \frac{[D^-][H^+]}{[DH]} \qquad (2)$$

$$[D^-] = Ka\frac{[DH]}{[H^+]} \qquad (3)$$

The total amount of drug in solution is the sum of the ionized form $[D^-]$ and the un-ionized form [DH]. The equation for total solubility, S_T, therefore can be written as:

$$S_T = [DH] + [D^-]$$

$$= [DH] + Ka\frac{[DH]}{[H^+]} \qquad (4)$$

since DH has previously been defined as equal to Ks:

$$S_T = Ks + Ks\frac{Ka}{[H^+]} = Ks\left(1 + \frac{Ka}{[H^+]}\right) \qquad (5)$$

This equation is a most useful one for determining the total solubility of a weak acid at a specific hydrogen ion concentration. Since the question most frequently asked is "What must the pH of the formulation be to maintain X amount of drug in solution?" a modified form of equation (4) is frequently useful:

$$S_T - Ks = \frac{KsKa}{[H^+]} \qquad (6)$$

or

$$[H^+] = \frac{KsKa}{S_T - Ks} \qquad (7)$$

For example: What must the pH of an aqueous formulation be to maintain in solution 10 mg/ml of a weakly acidic drug, molecular weight (MW) = 200, $K = 1 \times 10^{-5}$, Ks = 0.001 M/L?

The desired molar $\left(\frac{g/L}{MW}\right)$ concentration of drug $= \frac{0.010 \times 1000}{200} = 0.05M.$

$$[H^+] = \frac{(1 \times 10^{-3})(1 \times 10^{-5})}{0.05 - 0.001} = \frac{1 \times 10^{-8}}{0.049}$$

$$[H^+] = 2.04 \times 10^{-7}$$

$$pH = 7 - \log 2.04 = 7 - 0.31$$

$$pH = 6.69$$

An equation that is useful for poorly soluble, weakly basic drugs can be similarly derived:

$$\text{DOH (solid)} \rightleftharpoons \text{DOH (solution)}$$
$$Ks = \text{DOH (solution)}$$

and DOH (solution) is equal to the solubility of the undissociated base in moles/liter.

The dissociation of the weak base can be written as:

$$\text{DOH (solution)} \rightleftharpoons D^+ + OH^-$$

$$Kb = \frac{[D^+][OH^-]}{\text{DOH (solution)}} \qquad (8)$$

$$D^+ = \frac{Kb[\text{DOH (solution)}]}{OH^-} \qquad (9)$$

The total solubility of the base, S_T, is the sum of the ionized form $[D^+]$ and the un-ionized form [DOH]:

$$S_T = [DOH] + [D^+]$$

$$= [DOH] + \frac{Kb[DOH]}{OH^-} \qquad (10)$$

$$S_T = Ks + \frac{KbKs}{OH^-} \qquad (11)$$

Since:

$$Kw = [H^+][OH^-]$$

$$[OH^-] = \frac{Kw}{H^+} \quad (6)$$

then:

$$S_T = Ks + \frac{KbKs}{\frac{Kw}{H^+}} = Ks + \frac{KsKb}{Kw}[H^+] \quad (12) \quad (7)$$

Rewriting to solve for $[H^+]$:

$$\frac{S_T}{[H^+]} - \frac{Ks}{[H^+]} = \frac{KsKb}{Kw} \quad (13)$$

or:

$$[H^+] = \frac{Kw}{KsKb}(S_T - Ks) \quad (14)$$

In practice, these equations hold reasonably well; however, there are limitations that the reader should be aware of:

1. The values for the solubility constant Ks and the dissociation constants Ka or Kb that are reported in the literature (or determined in preformulation studies) are usually for the drug in distilled water. These values may be considerably different in a pharmaceutical dosage form such as an elixir, which contains a high percentage of solids and cosolvents. In general, cosolvents such as alcohol or glycerin have the effect of increasing Ks and decreasing the dissociation constant, as shown in Figures 15-1 and 15-2.[2]
2. The equations assume little or no interactions between the solute and itself or between the solute and other formulation components. At low concentrations of solute (below several percent), this assumption is generally valid.

In selecting the pH environment for adequate solubility, several other factors should be considered. The pH that satisfies the solubility requirement must not conflict with other product requirements, such as stability and physiologic compatibility. In addition, if pH is critical to maintaining drug solubility, the system must be adequately buffered. The selection of a buffer must be consistent with the following criteria:

1. The buffer must have adequate capacity in the desired pH range.

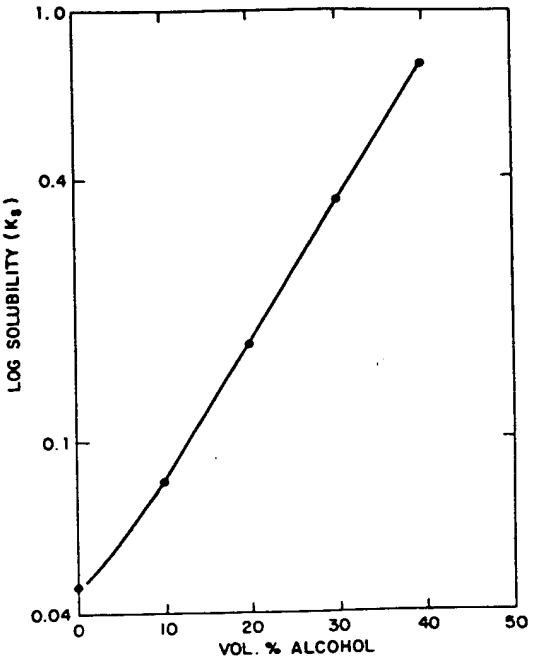

FIG. 15-1. *Effect of alcohol concentration on the solubility (Ks) of un-ionized sulfathiazole. (From Higuchi, T., Gupta, M., and Busse, L.W.: J. Am. Pharm. Assoc., Sci. Ed., 42:157, 1953.)*

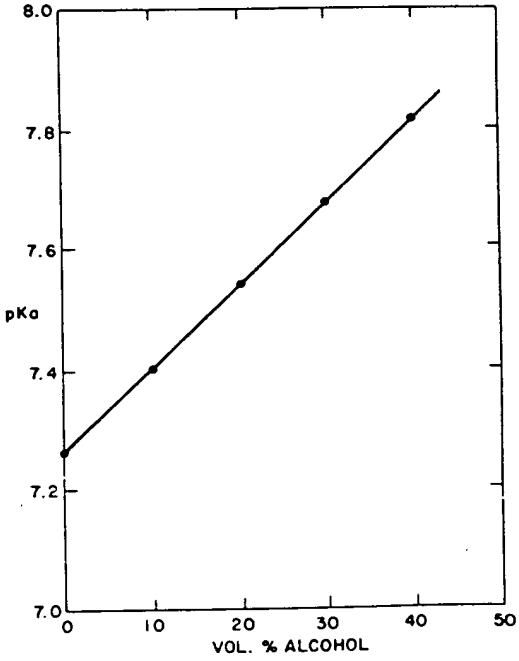

FIG. 15-2. *Effect of alcohol concentration on the dissociation constant (Ka) of sulfathiazole. (From Higuchi, T, Gupta, M., and Busse, L.W.: J. Am. Pharm. Assoc., Sci. Ed., 42:157, 1953.)*

2. The buffer must be biologically safe for the intended use.
3. The buffer should have little or no deleterious effect on the stability of the final product.
4. The buffer should permit acceptable flavoring and coloring of the product.

The first three points have been discussed by Windheuser[3]; the last needs no further elaboration. Figure 15-3 is a graphic representation of a number of pharmaceutically useful buffer systems and their effective buffer ranges. In general, a buffer system has adequate capacity within one pH unit of its pK. As an example of research in this area, Wang and Paruta have recently studied the effect of aqueous buffer systems and temperature on the solubility of commonly used barbiturates.[4,5] Basic information of this type is valuable to the formulator of liquid products.

For many drugs, a pH adjustment does not provide an appropriate means for effecting solution. In the case of very weak acids or bases, the required pH may be unacceptable in terms of physiologic considerations or owing to the effect of pH extremes on the stability of formulation adjuvants (such as sugars and flavors) or of the drug itself. The solubility of nonelectrolytes will, for all practical purposes, be unaffected by hydrogen ion concentration. In these cases, if solution is to be achieved, it must be done by the use of cosolvents, solubilization, complex phenomena, or in special circumstances, chemical modification of the drug to a more soluble derivative.

Cosolvency. Weak electrolytes and nonpolar molecules frequently have poor water solubility. Their solubility usually can be increased by the addition of a water-miscible solvent in which the drug has good solubility. This process is known as *cosolvency*, and the solvents used in combination to increase the solubility of the solute are known as *cosolvents*. The mechanism responsible for solubility enhancement through cosolvency is not clearly understood. It has been proposed that a cosolvent system works by reducing the interfacial tension between the predominately aqueous solutions and the hydrophobic solute.[6] Recent work supports the theory that amides adsorb to the solute at the interface with water, thereby diminishing the hydrophobic surface or solute/water interfacial tension.[7] As a result, the soluble hydrophilic portion of the amide cosolvent remains oriented toward the aqueous phase. Some workers have looked upon the phenomenon as a result of the independent solubility of the solute in each cosolvent. This is obviously a gross oversimplification, since the solubility of a substance in a blend of solvents is usually not equal to the value predicted on the basis of its solubility in the pure solvents. For example, undissociated phenobarbital has a solubility of approximately 1.2 g/L in water and 13 g/L in ethyl alcohol. The ratio of solvents, as well as pH, can alter solubility (Fig. 15-4).

Ethanol, sorbitol, glycerin, propylene glycol,

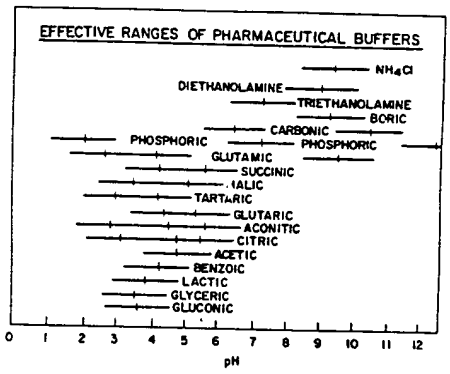

FIG. 15-3. *Commonly used pharmaceutical buffers and their effective buffer ranges. (From Windheuser, J.: Bull. Parenteral Drug Assoc., 17:1, 1963.)*

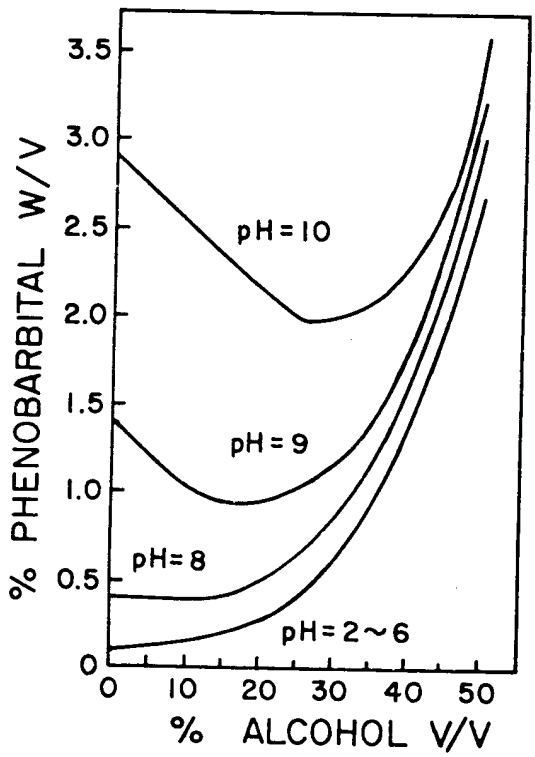

FIG. 15-4. *Interdependence of pH and alcohol concentration on the solubility of phenobarbital. (From Lin, K.S., Anschel, J., and Swartz, C.J.: Bull. Parenteral Drug Assoc., 25:44, 1971.)*